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THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF VEROCYTOTOXIN-2 RESISTANT VERO AND HELA 229 CELLS

by

Catherine Marie Moran

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
Master of Science

Department of Medical Microbiology and Infectious Diseases

EDMONTON, ALBERTA

Spring, 1989

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Abstract

The objective of this project was to investigate the mammalian cell receptor for Verocytotoxin-2 (VT-2 or Shiga-like toxin II) expressed by *Escherichia coli* O157:H$^-$. This was accomplished by characterizing alterations present in the cell surface of VT-2 resistant HeLa 229 and Vero cell clones. The clones were assigned to one of three groups based on their growth characteristics in the presence of VT-2. It was also determined that the clones exhibited varying degrees of resistance to Verocytotoxin-1 (VT-1 or Shiga-like toxin I).

The plasma membrane protein profile of the resistant cells was examined. Although differences were observed between membrane proteins of the VT-resistant and the parental types it was not possible to correlate the differences to the VT-resistant phenotype.

When the neutral glycolipid content of the VT-resistant cell membranes was examined, it was found that the cells which displayed the greatest degree of resistance to the toxin had the least amount of globotriasosylceramide (Gb_3) present; suggesting that this glycolipid may be a receptor for VT-2.

The ability of the VT-resistant clones to internalize ligands through receptor mediated endocytosis was also determined. It was found that one Vero cell clone, displayed a decreased rate of endosomal acidification, compared to the parental strain. Therefore, endosomal acidification may be required for VT entry into host cells.

A procedure was developed for the purification of VT-2 and VT-1. Therefore, it will now be possible to examine receptors in the toxin-resistant cells using a radioreceptor binding assay.

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Abbreviations

BSA	Bovine Serum Albumin
CD_{50}	the endpoint of the toxin titration in which 50% of the cell monolayer is killed at the end of 48 h incubation
EDTA	disodium ethylene diamine tetraacetic acid
EGF	Epidermal Growth Factor
EHEC	Enterohaemmorghaic *E. coli*
EPEC	Enteropathogenic *E. coli*
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
IEF	Isoelectric Focussing
K	Kilodalton
L	liter
LPS	lipopolysaccharide
MW	molecular weight
mL	milliliter
mg	milligram
µg	microgram
µL	microliter
µM	micromolar
mM	millimolar
M	molar
ng	nanogram
PBS	phosphate buffered saline
RME	receptor mediated endocytosis

rpm	revolutions per minute
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TCA	trichloracetic acid
TSB	Trypticase soy broth
VTEC	Verotoxigenic *Escherichia coli*
VT-1	Verocytotoxin 1 (Shiga-like Toxin 1)
VT-2	Verocytotoxin 2 (Shiga-like Toxin 2)

1. Introduction

1.1 Classification of the Diarrhoeagenic *Escherichia coli:*

It is well accepted now that certain serotypes of *Escherichia coli* which occur in the human intestinal tract may cause diarrheal type diseases, (Bray *et al.*, 1945; Drasar and Hill, 1974; Gorbach *et al.*, 1971; Levine *et al.*, 1983; Levine and Edelmen, 1984; Levine 1987; Penney *et al.*, 1986; Robins-Browne, 1987). However, in 1885 when Theodore Escherich first described this organism, it was not considered a human pathogen because it was found to be universally present in all human and animal intestinal tracts. It was not until 1945 when Bray *et al.* developed an antiserum which agglutinated certain strains of *E. coli* obtained from infants who had summer diarrhoea, that physicians began to re-evaluate the pathogenic potential of *E. coli*.

Subsequently, further evidence to establish *E. coli* as a diarrhoeal agent was established by Taylor *et al.* and Giles *et al.* in 1949 in two independent studies. They identified *E. coli* as the etiological agent in outbreaks of infantile diarrhoea in a hospital maternity unit and nursery in the United Kingdom. In the 1950's studies with human volunteers provided even more compelling evidence that certain *E. coli* strains were pathogenic (Konowalchuk *et al.*, 1977; Koya *et al.*, 1954a; 1954b). Koya and co-workers used *E. coli* (serogroup O111) in intubation studies to demonstrate that

clinical symptoms depended not only on the presence of the organism, but also upon its location in the gut. They found that when *E. coli* was present in the large intestine, no appreciable illness was induced, but when *E. coli* was present in the small intestine, a diarrhoeal-type illness developed.

At the same time that the association between *E. coli* and disease was being documented, Kaufmann was perfecting a scheme for serologically distinguishing between *E. coli* strains. The Kaufmann procedure was based on serological differences in surface antigens expressed by various strains of *E. coli*. The outer membrane of gram negative organisms contain lipopolysaccharide (LPS). LPS is composed of a complex lipid called lipid A. To this lipid is attached a polysaccharide composed of a core region and a terminal series of repeated saccharide units. The LPS polysaccharide moeity contains the different immunological epitopes which form the basis of the somatic O antigen serogrouping scheme. Each O serogroup can be further subdivided depending on the type of flagellar (H) antigen produced. The K antigens are a subject of controversy and at the present time are used to indicate only demonstrable surface acidic polysaccharide or protein fimbrial antigens (Orskov and Orskov, 1984). Today approximately 171 O serogroups and 56 H serotypes are recognized (Levine, 1987). In combination, these constitute the O:H system, which plays an important role in studies of epidemiology and pathogenesis of *E. coli* infections.

In addition to being enteric pathogens, *E. coli* may also be involved in disease processes at other anatomical sites: wound infections, urinary tract infections, septicemias, meningitis, peritonitis,and pneumonias (Klemm, 1985; Korhonen, *et al.*, 1986; Levine, 1987).

For convenience, the diarrhoeagenic *E. coli* may be divided into five groups based on their interaction with the intestinal mucosa, clinical symptoms of disease, epidemiology and, importantly, O serogrouping (Table 1). These groups are: enteropathogenic, enterotoxigenic, enteroinvasive, enterohaemmorhagic, and enteroadherent *E. coli* (Robins-Brown *et al.*, 1987).

1.2 Characteristics of the Enterohaemmorhagic *Escherichia coli* (EHEC):

For my thesis, I will not attempt to review all of the diarrhoeagenic *E. coli*. Instead I will restrict my discussion to the enterohaemmorrhagic strains also termed verotoxigenic *E. coli* (VTEC).

E. coli is capable of producing several different enterotoxins (O'Brien and Holmes, 1987) which are listed in Table 2. In 1977, Konowalchuk *et al.* discovered a cytotoxin present in the culture filtrates of a number of *E. coli* strains isolated from human diarrhoeal diseases, which were characterized by symptoms of grossly bloody stools. He also isolated cytotoxin from specimens of infected food products. The crude toxin differed from the heat-stable and

TABLE 1

Serogroups Associated with *E. coli* Diarrhoeal Diseases

Class of Diarrhoeagenic *E. coli*	Serogroups Commonly Associated with Disease
Classical Enteropathogenic	O26, O55, O86, O111, O119 O125, O126, O127, O128ab, O142, O18, O44, O112, O114
Enterotoxingenic	O6, O8, O15, O20, O25, O27, O27, O63, O78, O80 O85, O115, O128ac, O139, O148, O153, O159, O167
Enteroinvasive	O28ac, O29, O124, O136, O143, O144, O152, O164, O167
Enterohaemorrhagic (Verotoxigenic)	O157, O26, O111
Enteroadherent	O serogroups not yet defined

[Levine, 1987]

TABLE 2

Enterotoxins Produced by *E. coli*

Enterotoxin	Structure	MW	Mass of subunits α and β (Kd)	No. of subunits α and β	Immuno-genic	Bioassay	Mode of action
LT-I	protein	86000	28; 11.8	1; 5	yes	Y1 or CHO cells; rabbit ileal loops	Stimulate production of cAMP
LT-II	protein	ND	28; 11.8	ND	yes	Y1 or CHO cells	Stimulate production of cAMP
VT-1 (SLT-1)	protein	70000	32; 7.7	1; 5	yes	HeLa or Vero cell cytotoxicity; rabbit ileal loops; lethality to mice	Protein synthesis inactivation
VT-2 (SLT-II)	protein	ND[a]	ND[a]	ND[a]	yes	HeLa or Vero cell cytotoxicity; rabbit ileal loops; lethality to mice	ND
ST_a	polypeptide	2000			no	suckling mouse	Stimulate production of cGMP
ST_b	polypeptide	5000			no	weaned pig jejunal loops	ND

[a]not determined

[O'Brien *et al* ., 1988]

heat-labile enterotoxins produced by enterotoxigenic *E. coli*. They found that the toxin was cytotoxic for Vero cells, and hence named it Vero toxin (VT). At about the same time, Klipstein (1978) isolated toxin (from epidemics of acute diarrhoea in infants) which he termed "heat-labile-like-toxin". It is not clear exactly what toxin he was referring to, as he did not assay for cytotoxic effect in Vero cells, but it is felt that he too may have described VT (Klipstein *et al.*, 1978). At any rate, the ability to produce VT is closely associated with two clinical syndromes, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) which occur in patients infected with EHEC. These syndromes are described in more detail in Section 1.3.

Since its initial description by Konowalchuk, it has also been shown that VT is not just a single entity, but displays antigenic diversity (Karmali *et al.*, 1986, Scotland *et al.*, 1985). To date, at least two antigenitically diverse verocytotoxins have been identified: VT-1 and VT-2. A third toxin, pig oedema toxin is also believed to be a member of this group, but as yet has not received a VT designation (Gannon *et al.*, 1988; Linggood and Thompson, 1987).

VT-1 was originally described in *E. coli* strain H30, serotype O26:H11. This was shown to be biologically, physically and antigenitically similar to Shiga toxin produced by *Shigella dysenteriae* serotype I (O'Brien and Laveck, 1983; O'Brien *et al.*, 1982; O'Brien *et al.*, 1983).

For these reasons O'Brien *et al.* term VT-1 Shiga-like toxin (SLT).

VT-2 was first detected in *E. coli* O157:H7 in 1985 by Scotland *et al.* VT-2 is not neutralisized by anti-Shiga toxin antibodies. The immunological difference between these two toxins is supported by genetic studies which show that DNA probes for the two toxins do not cross hybridize (Willishaw *et al.*, 1985; Willishaw, 1987).

Pig oedema toxin is not neutralized by antitoxin against VT-1 or VT-2. Several *E. coli* serotypes are associated with oedema disease (O141:K85, K88ab; O141:K85; O139:K82; O138:K82) as well as some untypable strains.

1.3 Syndromes Related to EHEC:

As mentioned in Section 1.2, two clinical syndromes are recognized as a complication of verotoxigenic EHEC diarrhoea: haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). HC appears to be predominately associated with serotype O157:H7, although cases of bloody diarrhoea have been reported in patients infected with serotype O26:H11 (O'Brien *et al.*, 1983) as well as other *E. coli* serotypes such as O111 (Bitzan *et al.*, 1988). It is also felt that HC and HUS may not necessarily be separate entities, but one syndrome may develop into the other (Pai *et al.*, 1988; Morrison *et al.*, 1985). Typical symptoms of infected individuals include severe abdominal cramps, grossly bloody diarrhoea, which may be preceeded by watery diarrhoea, and

low or no fever (Padhye *et al.*, 1987; Spika *et al.*, 1986; Wells *et al.*, 1983). Upon examination by barium enema, the colon of EHEC-infected individuals displays a thumbprinting pattern. (Morrison *et al.*, 1985; Riley *et al.*, 1983). The clinical presentation of this illness differs from the dysentery described in shigellosis, *Campylobacter* enteritis, or enteroinvasive *E. coli* gastroenteritis by the lack of fever and the bloody discharge resembling lower gastrointestinal bleeding (Karmali *et al.*, 1983; Riley *et al.*, 1983; Gransden *et al.*, 1986).

HC was first documented in two separate outbreaks in Oregon and Michigan and the causative agent was subsequently isolated from contaminated beef patties (Riley *et al.*, 1983). From this outbreak *E. coli* O157:H7 was isolated from 4 out of six patients in Oregon and 6 out of 14 patients in Michigan. In addition, *Salmonella*, *Shigella*, *Campylobacter*, ova and parasites, *Yersinia entercolitica*, bacillus species, *Staphylococcus aureus*, enterotoxigenic and enteroinvasive *E. coli* were not detected in these patients. Control patients who presented with diarrhoea (not bloody) at the hospital during the same time interval were culture negative for *E. coli* O157:H7.

Further reports of *E. coli* O157:H7 in other centres (including the first report of an outbreak in Canada in a nursing home in Calgary by Pai *et al.* in 1984) soon followed. Before this time there had been reports of sporadic cases of HC in Canada by Johnson *et al.* (1983). Virtually all the

cases of *E. coli* O157:H7 occurring in Canada to 1983, including epidemic and sporadic cases, were reported to have identical plasmid profiles. This suggested that a single strain was responsible for all the illnesses occurring in Canada up to that point.

HUS was first described in 1955 by Gasser *et al.* There are two recognized forms of HUS. The familial form occurs in families where there is a genetic predisposition to the syndrome (Pirson *et al.*, 1987). In familial form, multiple episodes of HUS may occur throughout the life of the individual. The sporadic or epidemic form of HUS occurs as a result of an infectious agent and usually only one episode of HUS occurs (Drummond *et al.*, 1985). HUS is characterized by microangiopathic haemolytic anemia, thrombocytopenia and renal failure which is usually preceeded by a prodromal period of bloody diarrhoea (Gianantonio *et al.*, 1968). Besides this triad of symptoms, severe manifestations of the disease may include neurological involvement and thrombotic thrombocytopenic purpura (TTP) (Garella, 1987). HUS occurs in all age groups, but more commonly in infants and young children and is a major cause of renal failure in childhood (Gianantonio *et al.*, 1968). This syndrome has been associated with a number of infectious agents including viruses, bacteria (Ray *et al.*, 1970; Fong *et al.*, 1982; Kaplan and Drummond, 1978; Larke *et al.*, 1980), and microtatobiotes (Mettler, 1969). In 1983 Karmali *et al.* obtained evidence which associated HUS with EHEC infections

and shigellosis. Further, in this study, 24 out of 40 patients who were infected with EHEC and developed HUS had either VT or VT producing *E. coli* (VTEC) isolated from stool specimens. All of the VTEC were negative by the Sérèny test, which is the production of a purulent keratoconjunctivitis caused by the inoculation of virulent invasive organisms (enteroinvasive *E. coli*) into the conjunctival sac of a guinea pig; and negative for heat labile and heat stable toxins which are characteristic of enterotoxigenic *E. coli* infections. Subsequent to this study Karmali *et al.* (1985) and Scotland *et al.* (1988) established that VTEC are a major cause of the typical infectious form of HUS. It is now known that the VTEC and EHEC are basically the same organisms.

Although HUS is thought to occur after a diarrhoeal illness, rare extraintestinal VTEC infections may also precede HUS (Gransden *et al.*, 1985).

1.4 Animal Models for Verotoxigenic *E. coli* Disease:

Several animal models including chickens, mice, rabbits, calves and piglets have been investigated to see if they were appropriate to use in the study of the pathogenesis of VTEC infections in vivo (Farmer III and Davis, 1983; Potter *et al.*, 1985; Pai *et al.*, 1986). To study colonization and the diarrhoeal symptoms of VTEC diseases, the gnotobiotic piglet, the calf, and the infant rabbit appear to be relevant choices as they demonstrate symptoms which are similar to the syndromes exhibited by human hosts.

In gnotobiotic piglets, the bacteria cause an "attachment-and-effacing" effect on the microvilli similar to that seen in EPEC infections. Unlike the EPEC strains which attach to the entire intestine in piglets, VTEC colonize the caecum and colon. The symptoms produced in the piglet resemble that of oedema disease (Smith *et al.*, 1983) which attacks thriving pigs about one week after weaning. The major feature is progressive incoordination leading to partial or complete paralysis and death. Post-mortem examination shows oedema in various sites including the stomach wall and the mesentery of the colon (Linggood and Thompson, 1987).

In the calf challenged with serogroup O5 VT^+ the organisms caused "attachment-and-effacement" lesions in the ileum and large intestine (Francis *et al.*, 1986; Moxley and Francis, 1986). These lesions are somewhat similar to those observed in human HC.

When infant rabbits were inoculated intragastrically with *E. coli* O157:H7 it was observed that they consistently developed diarrhoea (Pai *et al.*, 1986; Sherman *et al.*, 1988). Mucosal damage was located mainly in the mid and distal colon, and characterized as "individual cell death" (Pai *et al.*, 1986). In the surface epithelium, mucin depletion and infiltration of neutrophils into the lamina propria and epithelium was observed. A similar observation was also made when the rabbits were fed a partially purified toxin preparation.

As yet, no satisfactory animal model has been developed for studying HUS after inoculation with either live bacteria or bacterial toxin.

1.5 Epidemiology of Verotoxigenic *E. coli* Infections:

In North America and Britain, VTEC infections associated with HC and HUS occur over a wide geographical area; are not strictly confined to an urban or rural population; and both sexes are affected equally. The majority of VTEC infections occur with serogroup O157:H7 EHEC and have been observed during the summer months. The most severe manifestations of VTEC infections occur in the extreme age groups (pediatric and geriatric populations) (Ryan *et al.*, 1986; Spika *et al.*, 1986; Karmali *et al.*, 1983).

It is generally accepted that food is the primary infectious source in man. Hamburger meat was implicated in at least two outbreaks of *E. coli* O157:H7 HC (Riley *et al.*, 1983; Ryan *et al.*, 1986). Unpasteurized milk has also been implicated in an outbreak of HC and two sporadic cases of HUS (Borczyk *et al.*, 1987; Moxley and Francis, 1986) and *E. coli* O157:H7 was isolated from healthy heifers on the farms associated with these outbreaks. Further evidence to establish cattle as a reservoir for *E. coli* O157:H7 was obtained in Argentina in 1977 (Martin, 1986). Examination of fresh meats and poultry from retail outlets for *E. coli* O157:H7 have shown that these organisms are present in 1.5-

3.7% of samples of beef, pork poultry and lamb (Doyle and Schoeni, 1987).

Recently (1987) a phage typing scheme has been proposed by Ahmed *et al.* for strains of *E. coli* O157:H7 which produce VT. Ninety-eight strains from Canada and the USA were typed and assigned to 14 phage types. It was found that the phage-typing results from outbreaks and sporadic cases correlated very closely with the epidemiological findings (Ahmed *et al.*, 1987).

1.6 Methods for Detecting Verocytotoxin:

The Vero cell cytotoxicity assay was the original method used to detect VT (Knowalchuk *et al.*, 1977). Cytotoxicity has also been demonstrated in HeLa cells (O'Brien *et al.*, 1983). To increase the sensitivity of the Vero cell assay, organisms obtained from clinical specimens were treated with Polymyxin B. This procedure released additional toxin from within the periplasmic space of the bacteria (Karmali *et al.*, 1985).

Detection of *E. coli* O157:H7 organisms in clinical samples may also be facilitated by their ability to utilize sorbital as a carbon source (Farmer III *et al.*, 1985) In addition to the plate media, Farmer also described a tube medium containing antisera to the H7 antigen which immobilizes the *E. coli* strains possessing this H antigen, and allows their rapid detection.

However, these assay systems are relatively non-specific, and are fairly time consuming. Tissue culture toxicity may require up to 48 hours to become apparent. A sandwich-type enzyme-linked immunosorbent assay has been devised to detect Shiga toxin as well as other related toxins (Kongmuang *et al.*, 1987). Although the assay is simple to perform and less time consuming than the cytoxicity assay, some strains which test positive in the verocytotoxicity assay do not give positive results by the Kongmuang procedure. Therefore, to ensure detection of clinical specimens which contain low concentrations of VT it is essential to retain the tissue culture systems until the sensitivity of other methods of detection can be improved.

1.7 Genetics of Verocytotoxin Production

The genes which control the production of VT-1 and VT-2 by *E. coli* are present in lysogenic bacteriophage. The first report of isolation of phage from a verocytotoxin-producing EHEC was from strain H19, serotype O26:H11 (Scotland *et al.*, 1983; Smith *et al.*, 1983). Further reports of the presence of phage in strains of EHEC occurred in serotypes O111, O119, O128, and O157 (Smith *et al.*, 1983; O'Brien *et al.*, 1984; Smith *et al.*, 1984; Karch *et al.*, 1987; Willishaw *et al.*, 1987). Verotoxin production has been shown to occur in other bacteria such as *Pseudomonas*, *Klebsiella*, *Vibrio* and *Salmonella* (Smith *et al.*, 1983; O'Brien, 1988). However, bacteriophage have not been isolated from the non-*E. coli*

producers, and similar to pig oedema toxin, it is felt that the genes encoding for toxin production may be present on the bacterial chromosome (Smith *et al.*, 1983; Marques *et al.*, 1987).

Two different morphologies of phage have been demonstrated from verotoxin producing EHEC. The first is a phage with an elongated hexagonal head with a non-contractile, flexible tail (Willishaw *et al.*, 1987). The second is a phage with a regular hexagonal head and a short contractile tail (Smith and Scotland, 1988).

In *E. coli* O26 thus far, only VT-1 has been detected and there has only been one type of phage particle isolated at any one time. In contrast, *E. coli* O157 may produce either VT-1 or VT-2 or both VT-1 and VT-2 (Strockbine *et al.*, 1986; Willishaw *et al.*, 1987). The type of VT produced is linked to the isolation of morphologically distinct phage which are believed to encode singly for each toxin.

1.8 Verocytotoxin Structure:

VT-1 and VT-2 are potent "A-B" structured toxins which, as mentioned earlier, are closely related to Shiga toxin produced by *Shigella dysenteriae* I. Verocytotoxins may be divided into two categories: [1] those which are immunologically cross-reactive with Shiga toxin (VT-1) and [2] those which do not immunologically cross react with Shiga toxin (VT-2 and pig oedema toxin).

Toxins of the Shiga family are composed of a single A subunit which is enzymatically active and multiple copies of the B subunit which mediate binding of the holotoxin to receptors in its target tissue (Donohue-Rolfe *et al.*, 1984; Eidels *et al.*, 1983; O'Brien *et al.*, 1980).

1.9 Verocytotoxin Function:

The A subunit of VT-1 is virtually identical to that of Shiga toxin differing only in one amino acid (Hovde *et al.*, 1988). However the A subunit of VT-2 only shares 56% amino acid homology with the A subunit of Shiga toxin and 58% nucleotide homology (Jackson *et al.*, 1987). Nonetheless, based on amino acid sequence data, all of these (VT-1, VT-2, and Shiga) toxins are very homologous with the A subunit of ricin, a potent plant toxin which is isolated from the castor bean (Endo and Tsurugi, 1987). The intracellular targets for ricin and the toxins of the Shiga family are the elements of the protein synthetic peptide-elongation cycle. Shiga toxin inhibits protein synthesis at two levels, the first by inhibiting binding of the aminoacyl t-RNA to the 60S ribosome and also by inhibiting the EF-1 dependent GTPase activity of the peptidyltransferase (Obrig *et al.*, 1987; Reisbig *et al.*, 1981). VT-1 inhibits eucaryotic protein synthesis by catalyzing the hydrolytic depurination of adenosine 4324 in the 28S rRNA of eukaryotic ribosomes (Hovde *et al.*, 1988). Evidence has also been offered by Hovde *et al.* (1988) that the active site on the A subunit of VT-1 contains a glutamic

acid residue at position 167. When glutamic acid 167 was changed to aspartic acid, a conservative amino acid substitution, the protein synthesis inhibitory action of the toxin was decreased by a factor of 1000, implying this residue may be present in the active site, or involved in maintaining the critical active conformation of the toxin.

Initially the effect of VT-2 on protein synthesis was described by Strockbine *et al.* (1986). At this time it was found that protein synthesis was inhibited but the mechanism was unknown. Subsequently, VT-2 has been shown to inhibit protein synthesis by blocking EF-1-dependent binding of amino acyl-tRNA to ribosomes (Ogasawara *et al.*, 1988). Even more recently, it was shown that the mechanism of VT-2 action at the level of protein synthesis was identical to that of VT-1, Shiga toxin, and the plant lectin, ricin (Endo *et al.*, 1988).

1.10 Verocytotoxin Tissue Receptor:

It has been demonstrated that the structure of Shiga toxin and the verotoxins are very similar (Endo and Tsurugi, 1987). This implies that these toxins may also share functional properties. Although the toxins possess similar enzymatic activities, the biochemical identity of the toxin receptors remains unclear.

There has been a degree of controversy concerning the identification of the functional mammalian tissue receptor for Shiga toxin. One group has presented evidence suggesting that the receptor is a glycolipid (Lindberg *et al.*, 1987).

Another group maintains that the functional receptor is a glycoprotein (Keusch and Jacewicz, 1977; Keusch *et al.*, 1987) But they do agree however, that the receptor contains the Galα1-4Gal sequence.

Since Shiga toxin and VT-1 have identical β-subunits (Calderwood *et al.*, 1987) it can be inferred that they should have similar binding specificities. Experimental work performed by Lingwood *et al.* (1987) implied that this was the case. The Lingwood procedure involved binding VT-1 to glycolipids immobilized on a silica gel matrix. In this system, it was demonstrated that VT-1 bound specifically to the neutral glycolipid globotriasosylceramide (Gb_3) containing the terminal Gal(α1-4)Gal sequence. This glycolipid is a relatively minor component of the mammalian Vero tissue culture cell line. To confirm the identity of the receptors, they used a mutant cell line which was resistant to α-interferon and cross-resistant to VT-1 and VT-2. Waddell *et al.* (1988) showed that reconstituting the cell line with liposomes containing Gb_3 was able to restore a degree of sensititivy of the mutant cell line to VT-2 (Lingwood *et al.*, 1987a; Waddell *et al.*, 1988).

1.11 Specific Aims of the Research:

The objectives of the present investigation were twofold: (1) in the light of the present controversy to further investigate receptors for VT-2, and (2) to purify to homogenity VT-1 and VT-2

To investigate receptors for VT-2, toxin-resistant Vero and HeLa 229 cell lines were selected. These cell lines were chosen because Vero and HeLa 229 cells are used as a biological system to detect the presence of VT (Scotland *et al.*, 1980; O'Brien *et al.*, 1983) in culture fluid supernatants and clinical specimens. Toxin-resistant cells were expected to have reduced numbers of surface receptors, defective toxin internalization mechanisms, and/or a defective intracellular target for the enzymatic activity. I wished to investigate VT-2 receptors by comparing parental cell plasma membrane components with those of VT-2 resistant cells expressing fewer VT-2 surface receptors. In addition, I wished to determine whether VT-1 and VT-2 shared receptors in the HeLa and Vero cell systems. In other words, I wished to determine whether resistant clones expressing reduced levels of VT-2 receptors would also be resistant to VT-1.

To purify the two toxins, a method adapted from Donohue-Rolfe *et al.*, 1984; Petric *et al.*, 1987; and Head *et al.*, 1988 utilizing ammonium sulfate precipitation, isoelectric focussing, and affinity chromatography on Sepharose Blue was used.

2. Materials and Methods

2.1 Cell Lines, Bacterial Strains and Monoclonal Antibodies Used in Experimental Procedures:

HeLa 229 (human epitheloid cervical carcinoma cell line; passage received approximately 146 from original tissue source), Vero (African green monkey kidney cells; passage received approximately 121 from original tissue source) and A 431 (a human epidermoid carcinoma cell line) tissue culture cells were obtained from the American Type Tissue Collection (Maryland, USA). A Chinese Hamster Ovary (CHO RPE 44) cell line which was resistant to diphtheria toxin, *Pseudomonas* Exotoxin A and ricin was kindly provided by Dr. J. Moehring, University of Vermont, Burlington, Vermont, USA).

The bacterial strains *E. coli* O157:H$^-$ (E32511, the prototypic VT-2 producer) and *E. coli* O26:H11 (which expresses VT-1), were obtained from Drs. Bernard Rowe and Sylvia Scotland, Colinsdale, England.

The monoclonal antibody, anti-SLT II directed against the β-subunit of VT-2 produced by *E. coli* O157:H7 was kindly provided by Dr. Nancy Strockbine (CDC, Atlanta, Georgia, USA). Hybridoma cells (CRL-1794) producing monoclonal antibody directed against the β-subunit of VT-1 was purchased from the American Type Tissue Culture Collection, Maryland, USA.

2.2 Selection Procedure for Obtaining VT-2 Resistant HeLa 229 and Vero Cells:

HeLa and Vero cells (6 x 10^4 cells/mL) were grown in Linbro 24-well tissue culture plates at 35°C in a humidified 5% CO_2 incubator in Eagles Minimal Essential Medium with Earle's salts and L-glutamine (MEM Gibco, California, USA). The growth medium was supplemented with 10% fetal bovine serum (FBS, Gibco) for HeLa cells and 3% FBS for Vero cells. The Vero and HeLa 229 cell lines which were used, were obtained from the ATCC, and were not subcloned in our laboratory before the selection procedure. When confluent monolayers were obtained (48 h), 100 µL of doubling dilutions of a filter sterilized Trypticase Soy Broth (Difco Laboratories, Detroit, Michigan, USA) grown polymyxin B-extracted *Escherichia coli* O157:H⁻ was added to each of the wells containing HeLa cells and undiluted toxin extract was added to the Vero cell wells. The culture plates were incubated at 35°C for an additional 48 hours. At this time, each well was washed with EDTA wash solution (see Appendix 5) to remove the dead and dying tissue culture cells, and the medium was replaced with fresh FBS-supplemented MEM. When discrete colonies of toxin-resistant tissue culture cells had formed, each well was washed with EDTA wash solution and the cells were dispersed with 0.1 mL of 0.25% trypsin (Gibco) and transferred to individual wells in a six-well tissue culture plate containing 4 mL of FBS-supplemented MEM and 50 µg/mL gentamicin (Gibco) to prevent

bacterial contamination of the monolayer. The six-well plates were incubated for approximately 5 days or, until discrete islands of cells had formed. In the case of HeLa cells the clones were isolated from each other using sterile glass cloning rings (10 mm x 10 mm). The bottom edge of the glass cloning rings (obtained from the University of Alberta Technical Services Department, Alberta, Canada) were lightly coated with a layer of sterile silicone vacuum grease (Fisher Scientific, Alberta, Canada). The cloning rings were placed over the clones with the aid of an inverted microscope. The isolated clones were then washed with EDTA wash solution and lifted with 0.25% trypsin. Finally, the cloned HeLa cells were grown in 24-well tissue culture plates.

When the cloned cell monolayers became confluent, they were lifted and transferred to 25-mL tissue culture flasks containing MEM supplemented with 10% FBS and 50 µg/mL gentamicin. Once the cells established themselves adequately, the FBS concentration in the growth medium was decreased to 5% and the toxin-containing solution, which was used to select the cells, was added to prevent toxin-sensitive cells from reestablishing themselves in the toxin resistant population.

The isolation of the toxin-resistant Vero cells differed from that of the HeLa cells slightly. Once the toxin-resistant Vero cells had produced small clones in the 6-well plates, the individual clones were isolated by a limiting

dilution procedure in 96-well plates. Wells containing individual cells were allowed to form islands. These islands were dispersed with trypsin and transferred to wells of a 24-well plate. Once confluent monolayers were formed, the Vero cells were maintained by the same procedure described for HeLa cells, with the exception that the MEM was supplemented with 3% FBS.

2.3 Testing VT-2 Resistant Cell lines for Mycoplasma Contamination:

The toxin-resistant cell lines were checked for mycoplasma contamination by the method of Robertson *et al.* (1975). In brief, the cell monolayers were treated with trypsin as described in Section 2.1, and the free floating cells were collected by centrifugation at 200 x g for 20 minutes. The cell pellets were suspended in B-broth (Appendix 10) and also plated out on a Standard agar plate (Appendix 10) and incubated for two weeks at 37°C in an anaerobic jar. Growth, if present in the broth, was detected by the indicator solution changing from chartreuse, to blue-green. Growth, if present on the agar plates, appeared as the typical fried-egg type colony.

2.4 Examination of the Growth Rate of Resistant Cells in the Presence of VT:

Briefly, this procedure was performed as described previously (Marnell *et al.*, 1984). One mL (5×10^3

cells/mL) of cells was added to each well in 24 well tissue culture plates in the prescence or absence of 100 µL of VT from *E. coli* O157:H⁻ or O26:H11 (titre 1:64). At various time intervals, the cells in selected wells were lifted with 0.25% trypsin and diluted to a final volume of 1 mL. The cell concentration was determined by counting the cells present in the 4 corners (white cell area) of a hemocytometer (Fisher Scientific, Edmonton, Alberta, Canada). Counting was done repeatedly until 168 hours, at which time the monolayers had become confluent. The assay was performed in triplicate.

2.5 Determination of the Plating Efficiency of VT-Resistant Cells:

The procedure was described by Marnell *et al.* (1984). One mL containing approximately 1 x 10^3 cells or 60 cells of each of the toxin-resistant HeLa cells or parental HeLa cells, and 200 cells and 100 cells of each of the resistant Vero cells or parental cells were added to a 25 mL tissue culture flask (in 5 mL total volume) in duplicate in the presence or absence of VT-1 or VT-2 (0.5 mL cell-free TSB supernatant solution containing VT). Each flask was allowed to incubate undisturbed for 2 weeks. At the end of the incubation period, the growth medium was discarded and the cells were fixed with methanol (Fisher Scientific). Each flask was incubated at 65°C to fix the cells to the plastic and subsequently stained with 0.5% crystal violet

(Fisher Scientific). The microcolonies of tissue culture cells which had formed were then counted and compared to the number of cells originally placed into the flask.

2.6 Preparation of FITC Labelled Epidermal Growth Factor:

Approximately 5 mg of EGF (a generous gift of Dr. Joseph Hayden, University of Calgary, Alberta, Canada) was weighed out and dissolved in 1 mL of 0.1 M sodium carbonate buffer (pH 9.0) in an aluminum foil-wrapped Eppendorff tube. It was necessary to prevent light from entering the tube in order to limit photobleaching of the fluorescein isothiocyanate (FITC). Two mg of dry FITC (Sigma Chemical Company, St. Louis, Missouri, USA) was added to the EGF solution. The mixture was then incubated on a rotary mixer for 1 hour at room temperature (The and Feltkamp, 1970). The FITC-EGF reaction mixture solution was then applied to a G-25 Sephadex gel filtration column (Pharmacia, Quebec, Canada, molecular weight seperation range 1000-5000), which had been equilibrated with PBS (see Appendix 1). The G-25 column was used to separate free FITC from the FITC-EGF complex (approximate MW 6,000). The column was eluted with PBS and 0.5 mL aliquots were collected. The fractions collected from the column containing the FITC-EGF (displaying green fluorescence) were pooled and stored at -70°C.

2.7 Binding of FITC-EGF to A 431 Cells:

A 431 cells, an epidermoid cell line which possesses large numbers of EGF receptors, were treated with 0.25% trypsin and suspended in MEM containing 10% FBS and 10% glucose (Fisher). Twenty µL of the cell suspension was dispensed into each well of a 10-well Linbro glass slide which had been sterilized by dry heat. The slide was incubated in a Petri plate for 3 hours at 37°C in a 5% CO_2 atmosphere. At the conclusion of the incubation period, 30 mL of FBS and glucose-supplemented MEM was gently dispensed into the Petri plate, so as to avoid disturbing the cells which had become attached to the glass slide, and the slides were incubated for an additional 24 hours. At this time, the slides were removed from the Petri plates, gently washed with ice cold PBS, and allowed to dry. The slides were then placed into a new, clean, dry Petri plate and 20 µL of unlabelled EGF (0.1 mg/mL in PBS + 1% BSA) was added to half the wells. After 15 minutes on ice, 20 µL of FITC-EGF was dispensed into all of the wells. The slides were incubated for an additional 45 minutes on ice. At the end of the incubation period, the slides were washed with ice-cold 1% BSA/PBS and fixed in cold acetone (Fisher, -20° C) for 10 minutes. The slides were air dried, mounted in glycerol/PBS, and examined with the fluorescence microscope at a fluorescence excitation wavelength of 493.5 nm.

2.8 Iodination of FITC-EGF:

FITC-EGF was iodinated by the Iodo-Gen procedure described by Spivak *et al.* (1977). This procedure labels amino acids like tyrosine which contain aromatic side chains. In brief, 10 MBq of Na[^{125}I] (Edmonton Radiopharmaceutical Centre, Alberta, Canada) was added to 100 µL of the FITC-EGF solution. The mixture was then placed into an Iodo-Gen-(1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) coated (40 µg Iodo-Gen Pierce Chemical Company, Rockford, USA) , 12 x 75 mm glass tube. The protein solution was exposed to iodine in the presence of Iodo-Gen for approximately 40 seconds. After the iodination procedure, the reaction mixture was filtered through a glass wool-plugged Pasteur pipet into a clean 12 x 75 mm tube. One hundred µL of cysteine (Sigma;[1 mg/mL in PBS]) solution was passed through the filter to reduce elemental iodine (I_2) and inhibit any further iodination. The percentage of incorporation of [^{125}I] counts into the FITC-EGF was determined by the trichloracetic acid (TCA) precipitation procedure described below.

Two hundred µL of PBS (containing 0.1% BSA) was added to the remainder of the mixture to prevent non-specific binding of labelled FITC-EGF to the Sephadex column. The mixture was then passed through a disposable Sephadex G-25 column (1 cm x 10 cm) equilibrated with PBS to separate the [^{125}I]-labelled protein from free radioactivity. Sixteen 0.5 mL fractions were collected and 5 µL aliquots of each fraction was counted

in an LKB Rackgamma II model 1270 gamma counter to determine the location of the fractions containing [^{125}I] labelled FITC-EGF. The fractions containing the labelled protein were pooled and used immediately in a binding experiment in A 431, Vero, and HeLa 229 cells (see Section 2.10).

2.9 Determination of [^{125}I] Incorporated into Protein by TCA Precipitation:

Ten µL of iodinated protein was mixed with 1 mL 1% BSA (Sigma) in PBS in a 12 x 75 mm glass test tube. This was done in duplicate. One-half mL of 10% TCA (Fisher Scientific) in water was added to one sample and 0.5 mL of PBS was added to the other. Both tubes were incubated on ice for at least 10 minutes. The tubes were then centrifuged at 700 x g for 10 minutes to sediment the precipitated protein in the tube containing TCA. The amount of radioactivity in the supernatant solution from each of the tubes was measured in the LKB Rackgamma II model 1270 gamma counter. The amount of radioactivity covalently coupled to protein was determined from the difference between the number of counts remaining in the TCA supernatant solutions and those in equivalent aliquots from the unprecipitated control tubes.

2.10 Determination of ^{125}I-FITC-EGF Binding to A 431, Vero, and HeLa 229 Cells:

A 431, Vero, and HeLa 229 cells were grown overnight in 24-well tissue culture plates as described in Sections 2.1

and 2.6. The next day, the culture media was removed from each well by aspiration and the cell monolayers were washed three times with 0.5 mL of ice-cold PBS. The monolayer was then covered with 0.2 mL of a solution of ice-chilled 1% BSA in PBS and placed on ice.

Ten µL of unlabelled EGF (0.1 mg/mL) was added to one half of the A 431, Vero, and HeLa 229 cell monolayers. The plate was allowed to incubate for 15 minutes on ice. Ten µL of ^{125}I-FITC-EGF was added to all the wells and the plate was incubated for an additional 45 minutes on ice.

At the end of the incubation period, each of the cell monolayer were washed three times with 0.5 mL of cold 1% BSA in PBS. After the final wash, 0.5 mL of 10% SDS in distilled H_2O was placed in each well, and the plate was incubated at 37°C for 15 minutes to completely solubilize the monolayers. The solubilized cells were transferred to 12 x 75 mm culture tubes and radioactivity was measured in the LKB Rackgamma II model 1270 gamma counter as described previously.

2.11 Endosome Acidification Assay Using FITC-EGF:

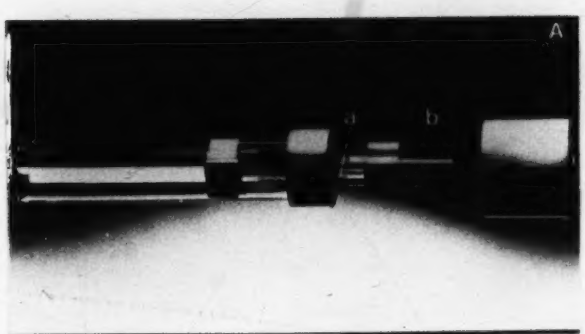
The endosomal acidification assay using viable tissue culture monolayers was originally described by Ohkuma and Poole (1978). This procedure depends on a shift in the fluorescence wavelength of FITC molecules when the enviromental pH decreases. Tissue culture cells (A 431, Vero, and HeLa 229) were lifted from the surface of the tissue culture flasks using 0.25% trypsin. The cells were

diluted to approximately 4.0×10^5 cells/mL and 2 mL of the suspension was dispensed into a plastic Leighton tube which contained an acid-washed, sterile glass coverslip (35 mm x 9 mm). The cells were then incubated in a humidified 5% CO_2 incubator for at least 24 hours. After the cells had formed a confluent monolayer, the coverslips were removed from the plastic support. Next the coverslip was gently washed, with ice-cold PBS. The coverslip was placed in the bottom half of a plastic Petri plate which had been previously cooled on ice. The coverslip was flooded with a 1/10 dilution of FITC-EGF in 1% BSA/PBS and incubated for 30 minutes in the dark. The incubation was performed in the dark and on ice for two reasons; (1) to avoid photobleaching of the FITC and, (2) to allow the FITC-EGF to bind to the surface of the tissue culture cells without being internalized during the incubation period. The coverslip was then removed from the Petri plate, washed with ice-cold 1% BSA/PBS, and inserted into a coverslip holder (Plate 1) which had been manufactured by Tyler Research Instruments Corporation, Edmonton, Alberta according to specifications of Ohkuma and Poole 1978. Briefly, the apparatus held the coverslip (9 x 15 mm) stationary at a 30° angle to the incident light beam to minimize reflection of incident light into the detector. The coverslip holder apparatus was placed in a cuvette (17 mm x 10 mm x 10 mm) which contained MEM pH 7.3 (prewarmed to 25°C). The apparatus which held the coverslip was also fitted with hollow metal tubing which allowed the addition of fresh media

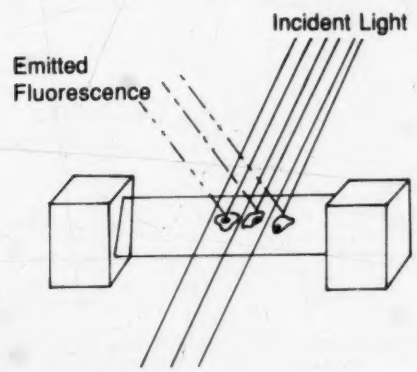
Plate 1: Photograph and Diagram of the Coverslip Holder for the Spectrofluorometer.

Panel A: The coverslip holder for the spectrofluorometer was constructed to fit into a 1 cm x 4 cm spectrofluorometer cuvette. The holder was able to hold a 9 x 15 mm coverslip (a). The holder was constructed out of hollow metal tubing (b) to allow the addition of media or reagents during the fluoresence determination without the apparatus being removed from the spectrofluorometer.

Panel B: The coverslip was held at a 30° angle to the incident light beam to minimize light scatter. The emitted fluoresence from the cells was measured at a 90° angle to the incident light beam.



B



or other reagents to the cuvette without the necessity of removing the coverslip holder from the cuvette or spectrofluormeter. The detector of the Turner Model 430 spectrofluormeter is fixed at an angle of 90° to the incident light source. The decrease in fluorescence as the FITC-EGF was internalized into the acidic endosomal vesicles was monitored continuously for 30 minutes at constant temperature maintained by circulating water from a Haake F3 Digital water bath through tubing lining the cuvette holder in the spectrofluorometer.

The FITC molecule is susceptible to photobleaching when exposed to light. In my experimental design to measure endosomal acidification, I monitored the decrease in fluorescence as the FITC-EGF entered the the endosomal compartment and the pH of the endosome fell. To control for the decrease in fluorescence due to the photobleaching reaction three experimental controls were performed. The first control employed identical coverslips one of which was fixed in acetone (-20°C) for five minutes prior to installation in the spectrofluorometer. The FITC-EGF was fixed to the cells and was not able to be internalized, therefore any decrease in fluorescence occuring in the acetone-fixed cells when compared to the unfixed coverslip, would be due to the photobleaching of the FITC molecule, and not due to the decrease in pH in the acidic enviroment of the endosome. A second control utilized monensin (Sigma), an inhibitor of endosomal acidification. A third control was

also performed with Chinese Hamster Ovary cells which were resistant to diphtheria toxin, ricin, and *Pseudomonas* Exotoxin A through an inability to acidify their endosomal compartments and thus allow penetration of the toxin to its target molecule (Moehring and Moehring, 1983).

2.12 Surface Iodination of Vero and HeLa VT-Resistant Tissue Culture Cells:

Vero and HeLa 229 cells were grown in 25-cm^2 tissue culture flasks as described earlier. The cell monolayers were then washed with 5 mL of lifting buffer (see Appendix 6). Next, an additional 5 mL of lifting buffer was added, and the flask was incubated at 37°C to allow the cell monolayer to detach from the plastic surface.

The free floating cells were suspended in 10 mL of PBS and sedimented by centrifugation (10 minutes, 200 x g at room temperature). The supernatant solution was discarded and the procedure was repeated once. The resulting cell pellet was suspended in 100 µL of PBS. The cell suspensions were iodinated in Iodo-Gen coated tubes as described above. Briefly, the cell suspensions were placed into an Iodo-Gen-coated 12 x 75 mm test tube. Approximately 10 MBq of Na[^{125}I] was added and the iodination reaction was allowed to proceed for 5 minutes with gentle, constant mixing. At the end of this time, 200 µL of 1 mg/mL cysteine in PBS was added and the reaction tubes were allowed to sit for an additional 2 minutes. The reaction mixtures were then removed from the

tubes, placed into 15-mL conical, plastic centrifuge tubes containing 10 mL of PBS, and centrifuged for 10 minutes at 700 x g. This washing procedure was repeated at least three times, to remove free iodine which had not been incorporated into cell proteins. The ^{125}I-labelled cells were then analyzed by SDS-PAGE using 7.5%-15% linear gradient acrylamide gels (see Appendix 4).

2.13 Preparation of Membranes from HeLa 229 and Vero Cells for Analysis of Proteins by SDS-Polyacrylamide Gel Electrophoresis:

The parental and toxin resistant HeLa 229 and Vero cells were grown in 25 cm^2 tissue culture flasks (approximately 1 x 10^6 cells/mL). Confluent monolayers were then lifted from the surface of the plastic using the lifting buffer described in Appendix 6. The cells were washed 3 times by centrifugation as described previously in Section 2.11 and at the end of the washing procedure, the cell pellet was suspended in 200 µL of ice-cold lysis buffer (see Appendix 6) containing 1 mg/mL of aprotinin and 1 mg/mL of $\alpha_2$macroglobulin (Boehringer Mannheim, Quebec, Canada) to inhibit proteolytic activity. Each cell pellet was then sonicated in a Branson model 220 sonicator water bath for 3 minutes. Next, the cell pellets were transferred to Eppendorf tubes, and centrifuged for 3 minutes in an Eppendorf Model 5413 table top centrifuge to sediment the cell membranes. The cell membranes were stored at -70° C until use.

2.14 Procedure Used for Labelling Tissue Culture Cell Glycolipids with ^{14}C-Serine:

This procedure was performed as described by Lingwood *et al.* (1987). Vero and HeLa 229 cells were grown in 75 cm^2 plastic tissue culture flasks, and allowed to incubate for approximately 24 hours. The tissue culture media was then decanted and each monolayer was washed 3 times with 10 mL of EDTA wash solution (see Appendix 5) to remove any serum or other media components which might interfere with the subsequent uptake of ^{14}C-serine. The ^{14}C-serine (Amersham, Ontario, Canada) was added to serum free media at a final concentration of 1 µCi/mL. The monolayers were incubated in the presence of ^{14}C-serine for 3 hours at 37°C in a 5% CO_2 atmosphere. At the end of this incubation period, MEM containing 3% FBS and 1 µCi/mL ^{14}C-serine was added to the monolayers to give a final volume of 10 mL. The monolayers were then allowed to incubate for an additional 48 hours.

2.15 Extraction of ^{14}C-Labelled Neutral Glycolipids from HeLa and Vero Cells:

This procedure was also performed as described by Lingwood *et al.* (1987). The confluent monolayers, which had been labelled with ^{14}C-serine, were lifted with 0.25% trypsin as described previously. Once the monolayers had lifted from the plastic, the cells were washed three times with PBS in a sterile 15-mL conical plastic centrifuge tube as described in

Section 2.11. After the final wash, the cell pellets were suspended in 1 mL PBS. The cell suspensions were then added to 20 mL of chloroform:methanol (2:1) in a 100-mL seperatory funnel. The glycolipids were extracted and the organic layer was collected. The pellet was extracted once more with 20 mL of the chloroform:methanol solution and the organic layer was collected and pooled with the first extract. The pooled fractions were saponified with 0.1 N NaOH/methanol at 37°C overnight. The saponified aliquots were flash evaporated and the residues were dissolved in 0.5 mL chloroform:methanol.

2.16 Thin-Layer Chromatography of Neutral Glycolipids:

Glycolipids were separated by ascending thin-layer chromatography using Polygram Sil G Thin Layer Chromatography plates (Brinkman, Ontario, Canada). The developing solution used was chloroform:methanol:water (65:25:4). The ^{14}C-labelled glycolipids were detected by autoradiography and the glycolipid standards (Biocarb, Quebec, Canada) were detected by exposing the plate to iodine vapour. The identity of the ^{14}C-labelled glycolipids was determined by comparing the relative mobility of the spots detected on the autoradiograms with that of authentic glycolipid standards.

2.17 Detection of Immunoreactive VT-1 and VT-2 in Cell-Free Culture Media from *E. coli* O157:H$^-$ and *E. coli* O26:H11:

The ELISA procedure was performed according to the method of Engvall *et al.* (1971). Briefly, cultures of *E.*

coli O157:H⁻ and *E. coli* O26:H11 were grown in TSB at 37°C with shaking overnight. The bacterial cells were then harvested by centrifugation at 10,000 x g for 20 minutes at 4°C. The bacterial cells were suspended in PBS, extracted with 0.1 mg/mL polymxin B, precipitated with 40% ammonium sulfate, dialyzed against 4 L of PBS and finally concentrated with an amicon filtration unit using a YM10 filter (Millipore, Bedford, Maryland, USA; [1000 molecular weight cutoff]). Lowry protein assays (Appendix 2) were performed on each crude toxin extract (antigen) and the protein concentration was adjusted to approximately 50 mg/mL in each sample.

Two hundred µL of coating buffer (Appendix 7) was dispensed into each well in a 96-well ELISA plate. Ten µL of the appropriate antigen was added into the coating buffer. The plate was incubated overnight at 4°C to allow the protein to bind to the wells. Each well of the plate was emptied, and washed three times with PBS-Tween (Appendix 7) dispensed from a squirt bottle. The plate was incubated at room temperature for 3 minutes between each wash.

Serial 1/10 dilutions of the monoclonal antisera were prepared in 1% BSA/PBS-Tween (Appendix 7). Two hundred µL of each dilution was added to wells containing antigen and incubated for 2 hours at room temperature. The plate was then washed three times with PBS-Tween solution as described above. Two hundred µL of the diluted secondary antibody, goat-anti-mouse IgG conjugated to alkaline phosphatase was

then dispensed into each well and the plate was incubated for an additional 2 hours at room temperature. Alkaline phosphatase substrate tablets (Sigma) were dissolved in diethanolamine buffer (Appendix 7). The wells of the ELISA plate were again washed with PBS-Tween, and 200 µL of the substrate solution was dispensed into each well. Colour development was allowed to proceed at room temperature until the well displaying maximum colour reached an absorbance of 2.000 at a wavelength of 405 nm in a Titertek Multiscan MC ELISA scanner. The titre of the antisera was defined as the reciprocal of the antisera dilution which produced an absorbance of 0.050 when the colour development endpoint was reached.

2.18 Procedure for Preparing *Escherichia coli* O157:H$^-$ and *Escherichia coli* O26:H11 Growth Curves and Toxin Titres:

Duplicate flasks containing 1 L of TSB (Difco) supplemented with 0.2 µg/mL of mitomycin C (Sigma) were inoculated with 50 mL of an overnight static TSB culture of either *E. coli* O157:H$^-$ or *E. coli* O26:H11 (both strains were obtained from Sylvia Scotland and Bernard Rowe, Public Heatlth Laboratory Services, London, England). The cultures were then incubated at 37°C with shaking. One mL aliquots were removed aeseptically at designated time intervals and the turbidity of the samples was determined at a wavelength of A 600 nm using a Gilford Model 250 Spectrophotometer to determine the rate of accumulation of bacterial cells.

Subsequently, the 1 mL sample was extracted with Polymyxin B by adding 100 µL of a 1.0 mg/mL solution of Polymyxin B and incubating the mixture at 37°C for 30 minutes. Next, the cell debris in the extracts was removed by centrifugation in an Eppendorf table top Model 5413 centrifuge for three minutes. The supernatant solution was filter sterilized through a Millex-GV 0.22 µm pore size membrane filter (Millipore) and the amount of toxin present was determined with the Vero cell assay. Briefly, 20 µL of two fold serial dilutions of the supernatant solutions were applied to confluent Vero cell monolayers grown in 3% FBS supplemented MEM. The plate was allowed to incubate for 48 hours and the cytopathic effect (CPE) in the monolayer was determined. The endpoint of toxin produced by the bacteria was defined as the reciprocal of the dilution of the extracted toxin required to kill 50% of the cell monolayer during the 48 hour incubation period (CD_{50}).

2.19 Determination of the Minimum Inhibitory Concentration (MIC) of Mitomycin C on Growth of *E. coli* O157:H$^-$ and O26:H11:

Petric *et al.* (1987) found that mitomycin C stimulated production of VT-1 in broth culture. To see if mitomycin C would increase the yield of VT-2, I performed an experiment to determine the MIC of mitomycin C and find a concentration which would not inhibit bacterial growth, but would cause the production of an increased amount of toxin.

Serial dilutions of mitomycin C were made spanning the concentration range of 1 mg/mL to 0.1 µg/mL. A TSB culture of *E. coli* O157:H$^-$ was adjusted to the turbidity of a No. 1 McFarland Standard (3 x 10^8 organisms/mL) (see Appendix 9). One mL aliquots of the bacterial suspensions were then added to each of the culture tubes containing a 1.0 mL aliquot of the mitomycin C dilution in TSB. The minimum inhibitory concentration was the lowest concentration of mitomycin C which prevented bacterial growth during 12 hours of incubation at 37°C.

2.20 Procedure Used to Purify VT-2:

Toxin purification was performed using a modification of the method described by Head *et al.* (1988). A 12-litre TSB culture of *E. coli* O157:H$^-$ containing 0.2 µg/mL mitomycin C was grown in Fernbach Flasks (Bellco, New Jersey, USA) with shaking at 37°C for 6 hours. At this time, the bacterial cells were harvested by centrifugation (10,000 x g for 30 minutes at 4°C) in the Sorvall RC-SB refrigerated superspeed centrifuge. The cell pellets were collected, pooled, and the clear supernatant solution was discarded. The pooled cell pellets were washed once with ice cold PBS (see Appendix 1) and suspended in 25 mL PBS containing 0.1 mg/mL Polymyxin B (Sigma) to extract VT-2 from the periplasmic space of the bacterial cell (Karmali *et al.*, 1985). The cell suspension was incubated at 37°C for 30 minutes with intermittant mixing and then centrifuged (83,000 x g for 1 hour at 4°C) to

separate cell debris from the supernatant solution. The supernatant solution was then dialyzed against 0.01 M potassium phosphate buffer (pH 7.2) and applied to a 1.5 x 20 cm hydroxyappatite column, equilibrated with 0.01 M potassium phosphate buffer (pH 7.2). Next, the column was washed with 2 column volumes of the equilibration buffer and eluted with approximately 300 mL of 0.05 M potassium phosphate buffer (pH 7.2). Three mL fractions were collected. Each fraction was analyzed for the presence of protein (A_{280} on a Gilford model 250 Spectrophotometer) and cytotoxic activity in Vero cell monolayers as described earlier. Fractions which contained a high A_{280} and cytotoxic activity were pooled and a protein assay (see Appendix 2) was performed. The pooled fractions were diluted with double distilled deionized water. The protein concentration of the pooled fractions was adjusted to approximately 10 mg/mL in order to prevent overloading the isoelectric focussing cell. Next the extract was loaded into a Rotofor™ Preparative IEF cell (Bio-Rad, California, USA) and focussed for 5 hours using Biolyte Ampholyte (Bio-Rad) pH interval 6-8 at 12 watts constant power and the temperature was maintained at 4°C. The fractions which produced CPE in Vero cells were pooled. All of the fractions obtained by the isoelectric focussing (IEF) procedure were analyzed by SDS-PAGE using the Laemmli discontinuous buffer system and a 16% acrylamide separating gel. (Apendix 3). The resultant gels were silver stained (Appendix 8) and photographed. At each stage of the purification process a sample was saved and the

toxin titre, protein concentration, and protein profiles on SDS-PAGE were obtained. Next, the IEF fraction containing VT activity was passed through a Sepharose Blue Column (Pharmacia) which was equilibrated with 10 mM sodium phosphate buffer (pH 7.2). Toxin was eluted from the Blue Sepharose using 300 ml of 50 mM sodium phosphate buffer pH (7.2). Each fraction was analyzed using the Vero cell assay. Fractions which displayed cytotoxicity were subsequently pooled and examined using a 16 % discontinuous SDS-PAGE gel as described earlier.

2.21 Procedure Used to Purify VT-1:

The VT-1 purification scheme was performed using a modification of the method described by Petric *et al.* (1987). A 6 L TSB culture of *E. coli* O26:H11 supplemented with 0.2 µg/mL mitomycin C was grown in Fernbach flasks with shaking at 37°C for 6 hours. At this time, the bacterial cells were harvested by centrifugation (10,000 x g for 30 minutes at 4°C) in the Sorvall RC-SB refrigerated superspeed centrifuge. The cell pellets were collected, pooled, and the clear supernatant solution was discarded. The pooled cell pellets were washed once with ice cold PBS (see Appendix 1) to remove any residual TSB and subsequently suspended in 25 mL PBS containing 0.1 mg/mL Polymyxin B (Sigma) to extract VT-1 from the periplasmic space (Karmali *et al.*, 1985). The polymyxin-treated cell suspension was incubated at 37°C for 30 minutes with intermittant mixing. At the end of the incubation

period, the cell suspension was centrifuged (83,000 x g for 1 hour at 4°C) to separate cell debris from the supernatant solution. The supernatant solution was diluted with water, as described in the previous section, loaded into a Rotofor™ Preparative IEF cell and focussed using the same conditions described in the previous section. After focussing, fractions were collected and tested for cytotoxic activity in the Vero cell assay. The fractions which produced CPE in Vero cells were pooled. The pooled fractions were then passed through an immunoaffinity column which was made from a monoclonal antibody (CRL-1794) directed against the β-subunit of VT-1. The column preparation is described in Appendix 11. The toxin was eluted with a 3 M sodium thiocyanate solution. The fractions were dialyzed against 4 L PBS (see Appendix 1). Each fraction was analyzed using the Vero cell assay and also analyzed for protein content by measuring the A_{280} with a 1 cm light path quartz glass cuvette in a Gilford model 250 Spectrophotometer. Fractions which displayed CPE were subsequently pooled and dialyzed against 6 L of PBS and analyzed using a 16 % discontinuous SDS-PAGE gel (Appendix 3) and silver stained (Appendix 10).

2.22 Production of Ascites Fluid Containing Anti-VT-1 IgG Monoclonal Antibody:

Ten white, female Balb/c mice were injected intraperitoneally on day 1 with 0.5 mL Pristane (2, 6, 10, 14 tetramethylpentadecane). On day 6, each mouse was injected

intraperitoneally with approximately 5.0 x 10^6 CRL 1794 hybridoma cells (ATCC) producing anti VT-1 IgG antibodies. By day 10 each mouse had formed ascites fluid. On day 12, the mice were sacrificed by cervical dislocation and the ascites fluid was collected. The total volume of fluid obtained was approximately 10 mL.

2.23 Procedure used to Isolate IgG from Ascites Fluid:

The ascites fluid was applied to a Protein A-agarose column which had been equilibrated with PBS. The flow through fraction was recycled through the column three times and the bound material was eluted with 3 M sodium thiocyanate. Half mL fractions were collected and the absorbance of each fraction was determined at 280 nm using 3 M sodium thiocyanate as a blank. Fractions containing IgG were pooled and dialyzed against 4 L of 0.1 M sodium bicarbonate. The IgG was then stored at -70° C.

2.24 Verocytotoxin Assay in Fractions Containing Viable *E. coli* :

In some cases (e.g., analysis of numerous column fractions) it was necessary to analyze small samples for VT activity without filter sterilizing them. In these instances, Vero cell monolayers were prepared in 96-well tisssue culture plates. Next, 20 µL of the samples were added into the 200 µL of media covering the cell monolayers. The 96-well plates were then incubated at 37°C for 15

minutes. At the end of the incubation period, the culture medium was carefully removed from each well without disturbing the monolayer of cells. The medium was replaced with fresh MEM containing 3% FBS and 50 µg/mL gentamicin to inhibit the growth of any contaminating bacteria. The plates were then incubated for 48 hours at 37°C in a 5% CO_2 atmosphere. At the end of this time, the cytotoxic effect on the cell monolayers was determined using an inverted microscope.

2.25 Glycolipid Tube Binding Assay of VT-1 and VT-2:

The neutral glycolipids Gb_3 and ganglioside GM_1 (Biocarb) were each dissolved in chloroform:methanol (2:1) to form a solution of 5 mg/mL. Five µL of each suspension was added to 10 µL of chloroform:methanol (2:1) in chromic acid cleaned, glass 12 x 75 mm culture tubes. The tubes were then shaken with a vortex mixer at a low speed and the chloroform:methanol was evaporated under a stream of dry N_2 gas in such a manner that concentric rings of Gb_3 or GM_1 were deposited on the bottom of the culture tubes. One hundred µL of PBS + 1% BSA was added to the bottom of each tube. Ten µL of ^{125}I-labelled VT-1 or VT-2 was added to the PBS-BSA solution and the tubes were incubated at 4°C for 3 hours. At the end of the incubation period, each tube was washed three times with 0.5 mL cold PBS + 1% BSA. After the last wash, the tubes were counted in a LKB Rackgamma II model 1270 gamma counter to measure the amount of bound radioactivity. VT-1

and VT-2 were iodinated by the Iodo-Gen procedure described in Section 2.7.

3. Results

3.1 Initial Isolation of VT-2 Resistant Vero and HeLa 229 Cells:

Two different approaches were followed to isolate Verocytotoxin-2 resistant tissue culture cell clones. The first approach involved the selection of toxin resistant clones by exposing untreated tissue culture cells to filter-sterilized Trypticase Soy Broth supernatant fluids of VT-2 producing *E. coli* 0157:H$^-$ (CD_{50} 64). The second approach involved the use of chemical mutagenic agents to increase chances of obtaining the desired mutants.

In the second approach, tissue culture cells were exposed to nitrosoguanidine at concentrations ranging from 1 ng/mL to 10 µg/mL. However, I was unable to obtain viable tissue culture cells after treatment with nitrosoguanidine. Meanwhile, the first approach which involved selection of VT-2-resistant unmutated cells resulted in the isolation of several viable cells which I was able to nurture into toxin-resistant clones. Consequently, further attempts to obtain toxin-resistant clones by the nitrosguanidine procedure were abandoned.

From untreated cells I isolated six VT-2-resistant Vero cell clones and eight VT-2-resistant HeLa cell clones. One of the toxin-resistant HeLa cell clones (HeLa cm:6) could not be maintained. However, the remaining toxin-resistant clones

displayed stable growth and I wished to initiate characterization by examining their growth properties in the presence and absence of VT-2.

3.2 Growth Characteristics of Vero and HeLa Cells Cultured in the Presence of VT-1 and VT-2:

Growth curves were obtained by inoculating a fixed number of cells into 1 mL of growth medium in 24-well plates. At 24 hour intervals, the cells were counted to determine the number present. A second approach to characterizing growth was to determine the plating efficiency of the clones in the presence of VT-2. This was done by inoculating a small number of cells into 25 cm^2 tissue culture flasks and comparing the number of colonies present after 2 weeks incubation to the number of cells initially placed into the flasks. These two experiments were performed to compare the toxin resistance of the clones to the parental strain and to each other. The growth curves served to moniter the growth of the cells over a relatively short time span (1 week). In contrast, the plating efficiency experiments monitered resistance to the toxin over a longer time period. In addition to performing the growth experiments in the presence of VT-2, experiments were performed to determine the ability of the clones to survive in the presence of VT-1. Each growth curve was performed in triplicate, on two separate occasions. In the presence of VT-1 or VT-2, the parental HeLa and Vero cells displayed no growth during the course of

the experiment (Figures 1 and 2). In addition to the figures, the growth of all the VT-2 toxin-resistant Vero and HeLa cell clones is summerized in Tables 3 and 4. In contrast to the parental cells, all of the Vero and HeLa 229 VT-2 toxin-resistant clones displayed growth in the presence of VT-2 (CD_{50} 64 in parental Vero cells). It should be noted that wells containing toxin-resistant cell lines which did not appear to have grown at some of the time intervals, in fact contained viable cells, but the number of cells present was below the detection level (1000 cells/mL) of the hemocytometer. I arranged the VT-resistant cells into three groups based on their growth in culture in the presence of VT-2 (Table 5). Group I contained those clones displaying a high resistance to VT-2; Group II contained those displaying intermediate resistance to VT-2; and Group III contained those displaying low resistance to VT-2. The clones from both the HeLa and Vero cells which I selected for further characterization were from Group I, although I did choose a cell line, designated HeLa cm:3, to characterize because it appeared to be initially resistant to VT-2, but as the cells were exposed to VT-2, resistance appeared to be lost.

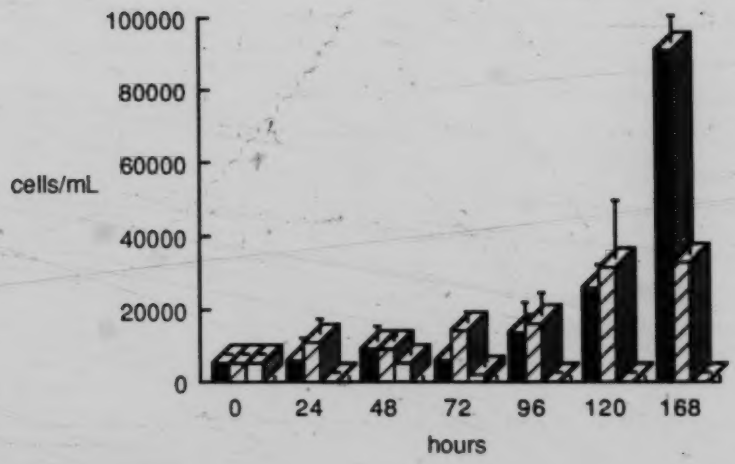
The results of the plating efficiency experiments are summerized in Table 6. The results of the plating efficiency experiments appeared to correlate well with the growth curves of the toxin-resistant cells with the exception of Vero cm:18. This clone displayed a relatively high resistance to the toxin in the growth curve experiment, but in the plating

Figure 1: The concentration of toxin used gave a CD_{50} of 64 in Vero cell monolayers. One hundred µL of this toxin extract was added to each well of the tissue culture cells containing 900 µL of medium.

Panel A. Bar Graphs Displaying Growth of VT-2 Resistant HeLa Cells (cm:1, solid bars), (cm:2, cross-hatched bars) and (cm:3, open bars) in the Presence of VT-2. The parental cell line did not display growth in the presence of VT-2. The error bars represent the standard deviation of the mean of the triplicate determinations

Panel B. Bar Graphs Displaying Growth of VT-2 Resistant HeLa cells (cm:1, solid bar), (cm:2, cross-hatched bar) and, (cm:3, open bars) in the presence of VT-1. The parental cells from which the VT-2 resistant cells were selected, displayed no growth in the presence of VT-1 (data not shown). The error bars represent the standard deviation of the triplicate determinations.

A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60000
40000
20000
0
cells/mL
0
24
48
72
96
120
168
hours

B

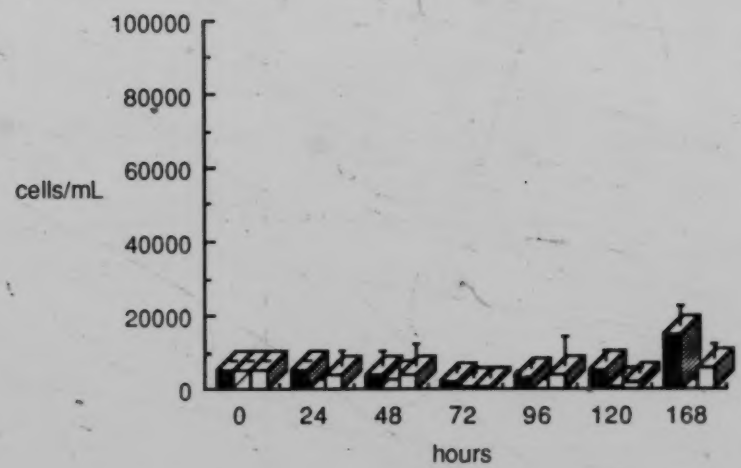
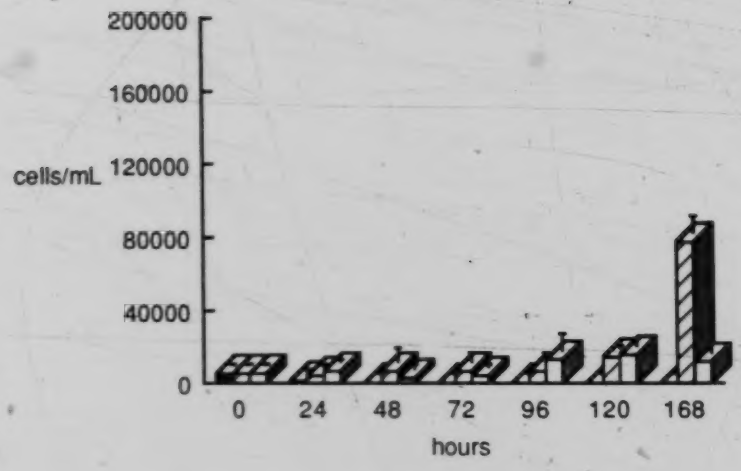

100000
80000
60000
40000
20000
0
cells/mL
0
24
48
72
96
120
168
hours

Figure 2: The concentration of toxin used gave a CD_{50} of 64 in Vero cell monolayers. One hundred µL of this toxin extract was added to each well of the tissue culture cells containing 900 µL of medium.

Panel A. Bar Graphs Displaying Growth of VT-2 Resistant Vero Cells in the Presence of VT-2. The parental Vero cell (solid bars) displayed no detectable growth in the presence of VT-2 after 24 hours. Vero (cm:17, open bars), Vero (cm:3, cross-hatched bars). Error bars represent the standard deviation of the mean of the triplicate determinations.

Panel B: Bar Graphs Displaying Growth of VT-2 Resistant Vero Cells Challenged with VT-1. Parental Vero cells (solid bars); Vero (cm:3, cross-hatched bars); Vero (cm:17, open bars) in the presence of VT-1. Error bars represent the standard deviation of the mean of the triplicate determinations.

A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160000
cells/mL 120000
80000
40000
0
0 24 48 72 96 120 168
hours

B

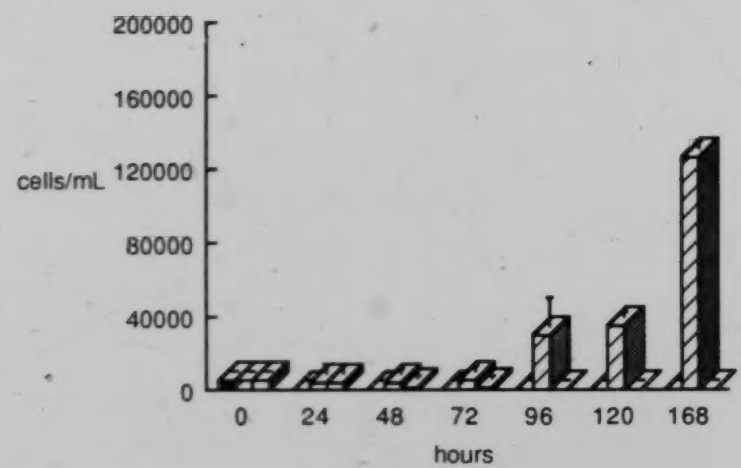

200000
160000
cells/mL 120000
80000
40000
0
0 24 48 72 96 120 168
hours

Table 3

Growth[a] of VT-Resistant HeLa Clones Challenged with VT-1[b] and VT-2[b]

Cell Line	VT-1 challenge (cell number x 10^3) 24 h	VT-1 challenge (cell number x 10^3) 168 h	VT-2 challenge (cell number x 10^3) 24 h	VT-2 challenge (cell number x 10^3) 168 h	Growth Control[c] (cell number x 10^3) 24 h	Growth Control[c] (cell number x 10^3) 168 h
HeLa 229	--[d]	--[d]	--[d]	--[d]	3.6 ± 5.0	252.0 ± 110.0
HeLa cm:1	5.0 ± 0	15.0 ± 5.0	6.0 ± 3.0	90.0 ± 7.0	4.0 ± 4.5	87.8 ± 41.5
HeLa cm:2	<1.0	<1.0	10.0 ± 3.0	330.0 ± 19.0	5.5 ± 5.0	18.2 ± 9.4
HeLa cm:3	4.2 ± 3.0	6.0 ± 4.0	<1.0	<1.0	1.0 ± 2.0	233.7 ± 34.0
HeLa cm:4	2.5 ± 1.0	5.0 ± 3.0	<1.0	<1.0	3.0 ± 2.9	370.0 ± 55.0
HeLa cm:5	2.5 ± 2.5	11.6 ± 3.0	2.0 ± 1.0	7.0 ± 5.0	5.0 ± 1.5	280.0 ± 72.0
HeLa cm:7	2.0 ± 1.0	7.5 ± 4.0	1.0 ± 0.5	1.7 ± 1.0	3.0 ± 1.5	630.0 ± 160.0
HeLa cm:8	2.5 ± 1.0	5.0 ± 5.0	<1.0	<1000	3.3 ± 5.0	350.0 ± 59.0

[a]Cell number represents the average of triplicate determinations ± the standard error of the mean.
[b]The quantity of VT-1 and VT-2 used was 100 µL of a polymyxin B-extracted supernatant which titred out to a CD_{50} of 64 in Vero cell monolayers.
[c]The number of cells present at the indicated times during the growth curve without the adddition of toxin to the growth medium.
[d]No growth detected at indicated time intervals

Table 4

Growth[a] of VT-Resistant Vero Clones Challenged with VT-1[b] and VT-2[b]

Cell Line	VT-1 challenge (cell number x 10^3) 24 h	VT-1 challenge (cell number x 10^3) 168 h	VT-2 challenge (cell number x 10^3) 24 h	VT-2 challenge (cell number x 10^3) 168 h	Growth Control[c] (cell number x 10^3) 24 h	Growth Control[c] (cell number x 10^3) 168 h
Vero	--[d]	--[d]	--[d]	--[d]	5.0 ± 2.5	45.5 ± 7.8
Vero cm:3	5.0 ± 5.0	130.0 ± 4.0	5.0 ± 1.0	77.5 ± 9.0	3.0 ± 2.5	330.0 ± 77.0
Vero cm:4	5.0 ± 4.0	150.0 ± 40.0	7.0 ± 1.0	335.0 ± 13.0	3.5 ± 3.9	370.0 ± 75.0
Vero cm:8	2.0 ± 1.0	<1.0	15.0 ± 6.0	16.5 ± 2.2	2.5 ± 4.0	240.0 ± 23.0
Vero cm:17	4.0 ± 4.0	<1.0	6.0 ± 1.0	11.7 ± 43.0	2.1 ± 5.0	220.0 ± 46.0
Vero cm:18	2.5 ± 2.5	38.0 ± 1.5	13.0 ± 8.0	210.0 ± 50.0	3.0 ± 2.5	280.0 ± 50.0
Vero cm:21	5.0 ± 4.0	5.5 ± 7.5	5.0 ± 4.0	49.0 ± 3.0	1.0 ± 1.0	313.0 ± 57.0

[a]Cell number represents the average of triplicate determinations ± the standard error of the mean.
[b]The quantity of VT-1 and VT-2 used was 100 µL of a polymyxin B-extracted supernatant which titred out to a CD_{50} of 64 in Vero cell monolayers.
[c]The number of cells present at the indicated times during the growth curve without the adddition of toxin to the growth medium.
[d]No growth detected at indicated time intervals

Table 5
Summary of Growth Characteristics of HeLa and Vero Cells

RESISTANCE CLASS		Growth[a] in Presence of VT-1[b]	VT-2[b]	CONTROL[c]	Plating Efficiency VT-1	VT-2
HIGH I	HeLa cm:1*	3x	15x	21x	2%	2%
	HeLa cm:2*	N.D.[d]	33x	33x	0.4%	17%
MODERATE II	HeLa cm:5	5x	4x	56x	55.8%	1.4%
	HeLa cm:7	4x	2x	200x	6.5%	4.6%
LOW III	HeLa cm:3*	1x	N.D.[d]	200x	4.2%	0%
	HeLa cm:4	2x	N.D.[d]	120x	0.7%	1.1%
	HeLa cm:8	2x	N.D.[d]	105x	1.0%	1.0%
HIGH I	Vero cm:3*	26x	16x	110x	0%	59.3%
	Vero cm:4	30x	48x	105x	5.7%	11.3%
	Vero cm:18	15x	16x	95x	0.9%	0%
Moderate II	Vero cm:17*	N.D.[d]	20x	105x	0.1%	0%
	Vero cm:21	1x	10x	300x	1.9%	0%
LOW III	Vero cm:8	N.D.[d]	N.D.[d]	95x	0.1%0.	6%

[a]The numbers presented refer to the increase (to the nearest integer) after 168 hours incubation. The data from which these calculations were made are presented in Tables 3 and 4.
[b]Plating efficiency=the number of colonies formed after two weeks incubation in the presence of VT-1 or VT-2 compared to the number of cells originally placed in the flask (x 100). The quantitiy of toxin used was 0.5 mL of a polymyixn B extracted VT-1 or VT-2 added to 4.5 mL of growth medium (CD_{50}=64 in Vero cell monolayers).
[c]The control column refers to the growth of the tissue culture cells in culture, under the same condition as those exposed to VT-1 and VT-2, but without the presence of VT.
[d]N. D. no growth detected by hemocytometer.
*Clones which were characterized further.

Table 6

Plating Efficiencies[a] of VT-2 Resistant HeLa and Vero Cell Clones in the Presence of VT-1 and VT-2

Cell Line	VT-1 Challenge	VT-2 Challenge
Parental HeLa	0%	0%
HeLa cm:1	2.0%	2.0%
HeLa cm:2	0.4%	16.5%
HeLa cm:3	4.2%	0%
HeLa cm:4	0.7%	1.1%
HeLa cm:5	55.8%	1.4%
HeLa cm:7	6.5%	4.6%
HeLa cm:8	1.0%	1.0%
Parental Vero	0%	0%
Vero cm:3	0%	59.3%
Vero cm:4	5.7%	11.3%
Vero cm:8	0.1%	0.6%
Vero cm:17	0.08%	0%
Vero cm:18	0.9%	0%
Vero cm:21	1.9%	0%

[a]Plating Efficiency is the percentage of inoculum which had formed colonies after 2 weeks incubation in the presence of toxin.

efficiency experiment, it did not appear to be resistant to VT.

Several of the clones displayed similar plating efficiencies in the presence of VT-1 and VT-2 (Table 6). For example, HeLa cm:1; cm:4; cm:7; cm:8; and Vero cm:4; cm:8; cm:17; and cm:21. This suggested that the mechanism of resistance to the two toxins was similar in these isolates. However, some of the isolates (HeLa cm:2; cm:3; cm:5; and Vero cm:3; and cm;18) displayed differential resistance to the two toxins, implying that there may be differences in the processing of the toxin either in the initial interaction of the toxin with the surface receptors, or in the subsequent steps of internalization and interaction of the toxin with its intracellular target. When the VT-2 resistant cells were examined on Giemsa stained slides, no significant differences ($p>0.2$) were detected in their size when compared to the parental strains (Tables 7 and 8), but some of the clones when exposed to VT-2 appeared to display a diffusely staining cytoplasm (Plates 2 and 3).

To make sure that the morphological variation I observed in the cell lines was due to their toxin-resistance and not due to an extraneous factor such as contamination by mycoplasma, I cultured each resistant cell line for mycoplasma (Robertson *et al.*, 1975). I did not isolate mycoplasma from any of my clones.

To determine which level(s) of toxin activity was/were blocked in the resistant cells, I wished to biochemically

Table 7

Determination of Average Size[a] of Parental and VT-Resistant HeLa Cells

	(+) VT-2	(-) VT-2
HeLa 229	--	33.98 x 18.82
HeLa cm:1	32.75 x 23.17	36.75 x 27.53
HeLa cm:2	46.86 x 29.14	36.76 x 22.82
HeLa cm:3	47.39 x 28.05	37.11 x 22.83
HeLa cm:4	43.25 x 26.67	35.72 x 22.66
HeLa cm:5	31.75 x 24.39	33.63 x 22.30
HeLa cm:7	40.25 x 28.40	42.51 x 24.92
HeLa cm:8	49.27 x 27.08	36.76 x 21.78

[a]Meaurements were made using a microscope eyepiece micrometer. The size of the cells is given in μm. The values presented represent the average of 14 determinations.

Table 8

Deterimination of Average Size[a] of Parental and VT-Resistant Vero Cells

	(+) VT-2	(-) VT-2
Vero Parent	--	50.35 x 34.32
Vero cm:3	51.57 x 28.23	41.99 x 17.61
Vero cm:4	50.52 x 18.82	52.61 x 25.27
Vero cm:8	50.35 x 28.23	49.83 x 24.57
Vero cm:17	44.43 x 26.84	47.56 x 22.48
Vero cm:18	47.21 x 21.96	45.82 x 20.91
Vero cm:21	57.14 x 17.61	54.71 x 24.75

[a]Meaurements were made using a microscope eyepiece micrometer. The size of the cells is given in μm. The values presented represent the average of 14 determinations.

Plate 2: Photographs of Giemsa-stained Parental HeLa 229 Cells, and Verocytotoxin-2 Resistant HeLa Cell Lines in the Absence and Presence of VT-2. Photographed at 48 h incubation (Magnification 400x)

A: Parental HeLa 229 Cells

B: HeLa cm:1 in the absence of VT-2

C: HeLa cm:1 in the presence of VT-2

D: HeLa cm:2 in the absence of VT-2

E: HeLa cm:2 in the presence of VT-2

F: HeLa cm:3 in the absence of VT-2

G: HeLa cm:3 in the presence of VT-2

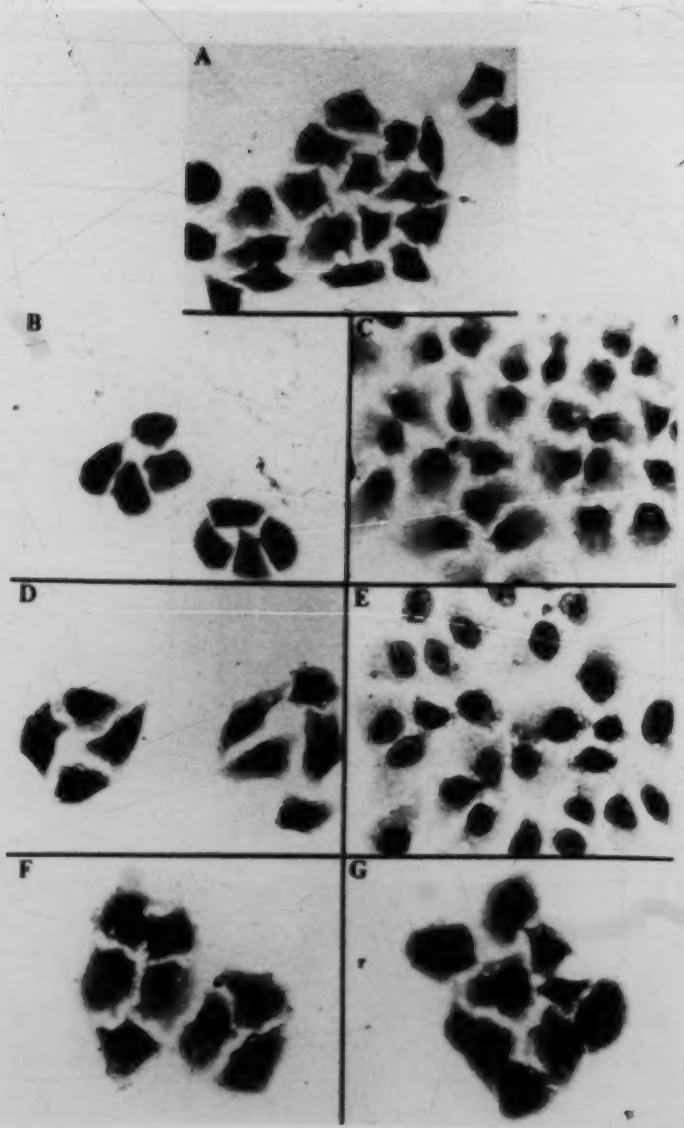

A
B
C
D
E
F
G

Plate 3: Photographs of Giemsa-stained Parental Vero cells and Verocytotoxin-2 Resistant Cell Lines in the Absence and Presence of VT-2. Photographed at 48 h incubation. (Magnification 400x)

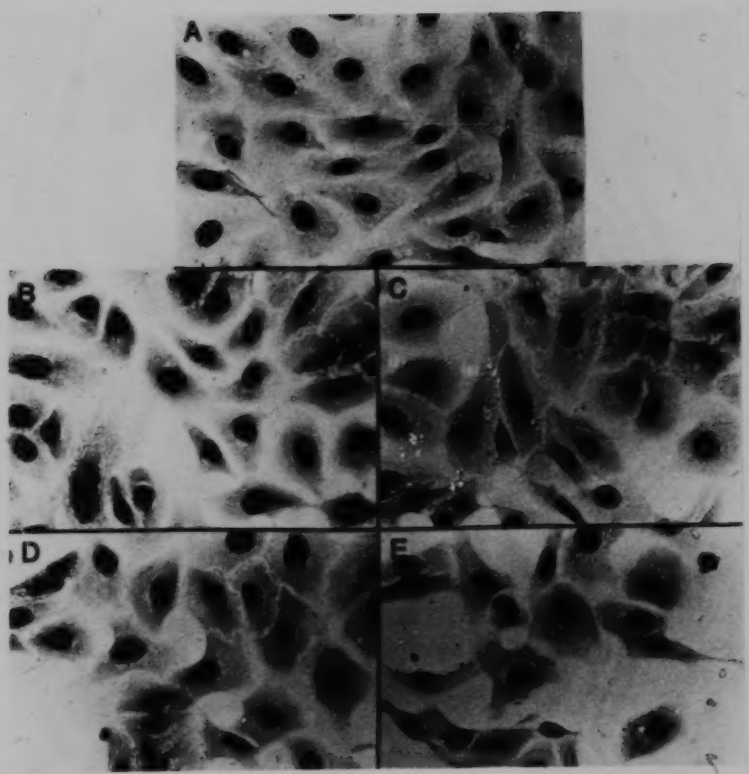
A: Parental Vero cells

B: Vero cm:3 cells in the absence of VT-2

C: Vero cm:3 cells in the presence of VT-2

D: Vero cm:17 cells in the absence of VT-2

E: Vero cm:17 cells in the presence of VT-2


A
B
C
D
E

characterize the clones. These studies were aimed at the level of the plasma membrane since I initially wished to investigate the VT-2 toxin receptor. However, I also wanted to examine the ability of the clones to acidify their endosomes during the process of receptor-mediated endocytosis (RME) as this process is a possible means of entry of the toxin into the cell.

3.3 Surface Exposed Membrane Protein Profiles of VT-2 Resistant HeLa and Vero Tissue Culture Cells Labelled with ^{125}I:

To characterize the toxin-resistant clones and the parental strains further, I used the SDS-PAGE procedure to compare the membrane proteins of the toxin-resistant strains HeLa cm:1; cm:2; and cm:3 with the parental HeLa 229 cell. Membrane proteins were identified by the Iodo-Gen catalyzed surface iodination technique (Plate 4). Comparison of membrane proteins in parental and toxin-resistant vero cells is shown in Plate 5.

When the HeLa cells were examined, I found that HeLa cm:3 lacked one band with a molecular weight of 280 K and one band with a molecular weight of 194 K. I also noticed the presence of an apparent increase in two protein bands with molecular weights of 41 K and 35 K in all three HeLa cell clones. When the Vero cells were examined it was noticed that the cells appeared to take up much less radioactivity than the HeLa cells. However, it was evident the VT-

Plate 4: Comparison of Parental and Toxin-Resistant HeLa Cell Plasma Membrane Proteins by SDS-PAGE

The cells were surface-labelled with ^{125}I as described in the materials and methods section. Samples of the iodinated cells were solubilized in Laemmli sample buffer and analyzed by SDS-PAGE in the presence of 2-mercaptoethanol. A linear 7.5%-15% acrylamide separating gel was used for the analysis. The positions of the protein standards are indicated by their molecular weights (x 10^3) on the left side of the photograph. The 250,000 molecular weight standard corresponds to the upper spectrin band in human erythrocyte membranes (Marchesi *et al.*, 1976).

Panel A. Autoradiogram exposed for 12 hours at -70°C.

Panel B: Autoradiogram exposed for 48 hours at -70°C.

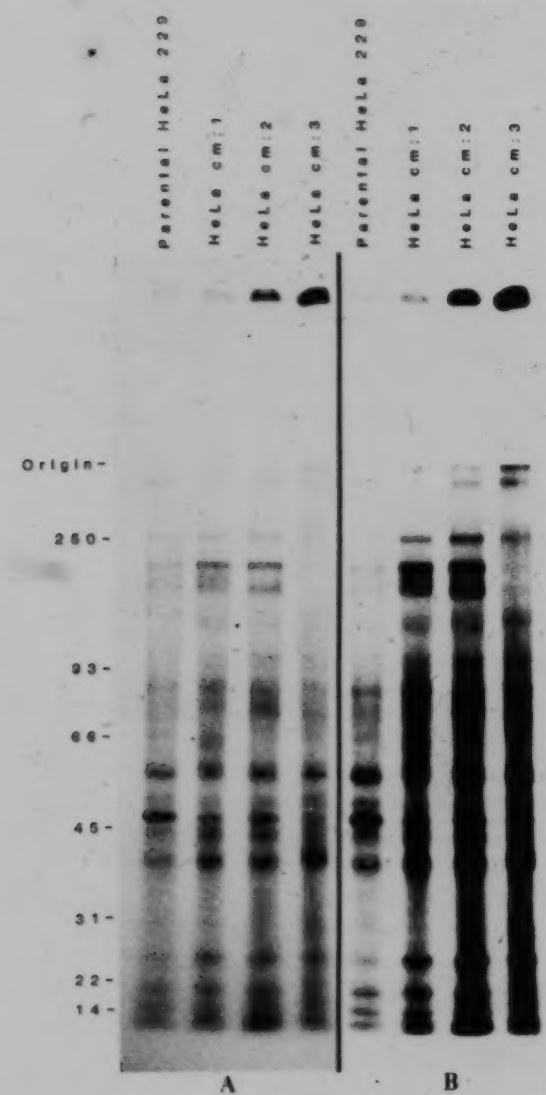
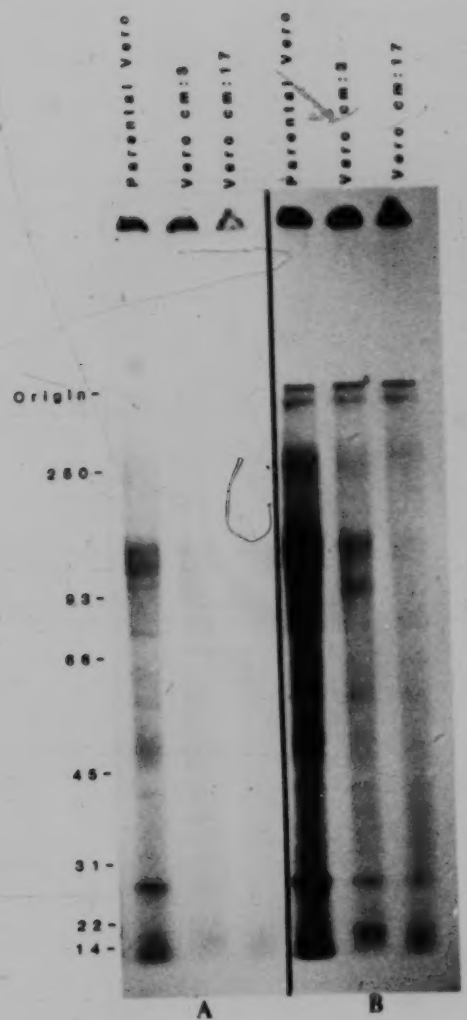

Parental HeLa 220
HeLa cm:1
HeLa cm:2
HeLa cm:3
Parental HeLa 220
HeLa cm:1
HeLa cm:2
HeLa cm:3
Origin-
250-
93-
66-
45-
31-
22-
14-
A
B

Plate 5: Comparison of Parental and Toxin-Resistant Vero Cell Plasma Membrane Proteins by SDS-PAGE

The cells were surface-labelled with ^{125}I as described in the Materials and Methods Section. Samples of the iodinated cells were solubilized in Laemmli sample buffer and analyzed by SDS-PAGE in the presence of 2-mercaptoethanol. A linear 7.5%-15% acrylamide separating gel was used for the analysis. The positions of the protein standards are indicated by their molecular weights (x 10^3) on the left side of the photograph. The 250,000 molecular weight standard corresponds to the upper spectrin band in human erythrocyte membranes (Marchesi *et al.*, 1976).

Panel A. Autoradiogram exposed for 12 hours at -70°C.

Panel B: Autoradiogram exposed for 48 hours at -70°C.


parental Vero
Vero cm:8
Vero cm:17
parental Vero
Vero cm:8
Vero cm:17
Origin
250
93
66
45
31
22
14
A
B

resistant Vero cell clones did not possess a protein band with a molecular weight of 93 K which was present in the parental cells. I also prepared whole cell lysates of each parental and toxin-resistant cell line and analyzed them by SDS-PAGE. When these gels were stained with Coomassie blue, no differences were noticed between the parental and toxin-resistant clones.

3.4 Analysis of Neutral Glycolipids in VT-Resistant HeLa and Vero Cells

Lingwood *et al.*, (1987) proposed that the receptor for Verocytotoxin 1 and 2 in mammalian cells is the neutral glycolipid, Gb_3. To see if the VT-resistant cells were deficient in this neutral glycolipid, I metabolically labelled the clones with ^{14}C-serine (Plate 6) and then compared the ratio of counts incorporated into the Gb_3 fraction with counts present in the remaining glycolipids. To determine which spot represented Gb_3, I analyzed a series of glycolipid standards on the same plate as the tissue culture cell extracts and also mixed authentic Gb_3 with each of our unknown samples. I found that the tissue culture cells which displayed the highest resistance to verocytotoxin had the greatest decrease in the amount of Gb_3 in comparison to the rest of the neutral glycolipids present in the sample (Table 9). The two clones HeLa cm:3 and Vero cm:17 which were the least resistant (see Table 5) of the clones

Plate 6: Autoradiograms of HeLa and Vero Cell Glycolipids Metabolically labelled with ^{14}C-serine and Analysed by Thin Layer Chromatography. The positions of the neutral glycolipid standards: cerebroside (**Cer**=Galβ1-4Cer and Glcβ1-4Cer), Lactosylceramide (**Lac**=Galβ1-4Glcβ1Cer), Globotriaosylceramide (**Gb_3**=Galα1-4Galβ1-4Glcβ1-1Cer), Globoside (**Gb_4**=GalNacβ1-3Galα1-4Galβ1-4Glcβ1-1Cer), and the Forssman glycolipid (**Gb_5**=GalNacα1-3GalNacβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) are indicated on the left side of the autoradiogram. The cerebroside standard migrated as a doublet at the position indicated (because it was a mixture of Gal and Glc). The number of counts of ^{14}C-serine incorporated into the Gb_3 spot were compared by ratio to the amount of ^{14}C-serine incorporated into each of the two glycolipid spots indicated by the asterix. Lane 1 HeLa 229; parental cells, Lane 2 HeLa cm:1; Lane 3 HeLa cm:2; Lane 4 HeLa cm:3; Lane 5 Vero parental cells; Lane 6 Vero cm:3; and Lane 7 Vero cm:17.

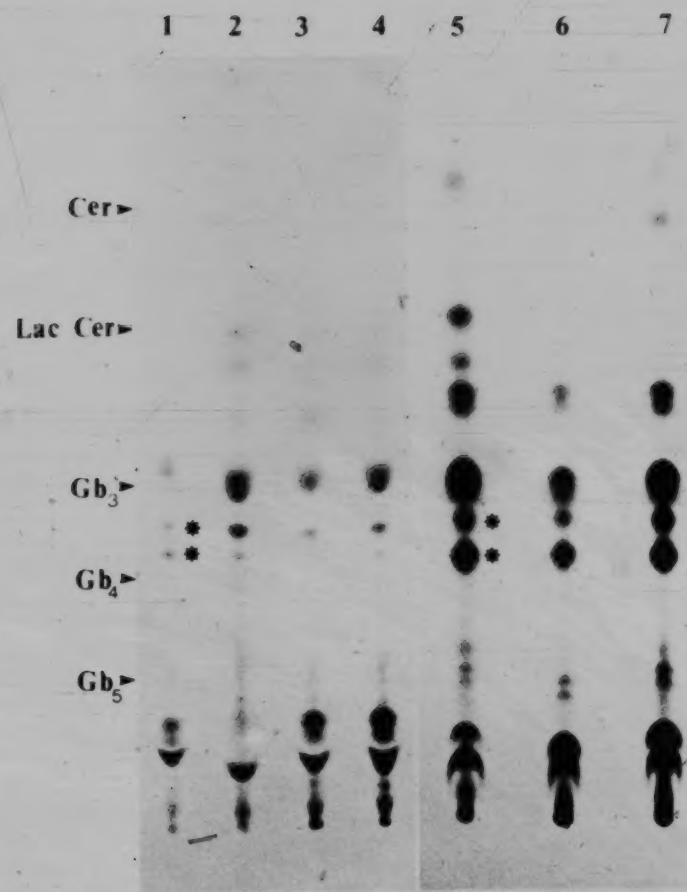

1
2
3
4
5
6
7
Cer
Lac Cer
Gb3
Gb4
Gb5

Table 9

Gb_3 Concentration of VT-2 Resistant Clones Relative to Other Neutral Glycolipids Present

Cell Line	Relative Gb_3[a] Concentration ± Standard Deviation	Resistance[b] to VT-1	Resistance[b] to VT-2	Cummulative Resistance Score
Parental HeLa 229	2.18 ± 0.15	--	--	4-
HeLa cm:1	1.02 ± 0.73	++	±+	3+
HeLa cm:2	1.27 ± 0.11	-+	±+	1+
HeLa cm:3	2.58 ± 0.69	±+	--	1-
Parental Vero	0.43 ± 0.22	--	--	4-
Vero cm:3	0.34 ± 0.27	+-	++	3+
Vero cm:17	0.43 ± 0.37	--	+-	2-

[a]Denotes relative concentration of Gb_3 to the neutral glycolipids indicated by the asterix(see plate 6) (n=3). Standard Gb_3 was mixed with ^{14}C-labelled glycolipids. The spot corresponding to Gb_3 was identified by exposing the plates to iodine vapour. These were cut out of the silica gel and the cpm incorporated was determined in a scintillation counter.

[b]A plus sign signifies resistance, a minus sign signifies that the clone was sensitive. In each column the first sign refers to results obtained in the growth curve experiments (see Tables 3 and 4) The second sign refers to the plating efficiency result (Table 6). The cumulative score refers to the arithmetic sum of the signs of the resistance columns.

contained essentially the same amount of Gb_3 as the parental strains. This suggested that the mechanism of resistance in HeLa cm:3 and Vero cm:17 isolates might be at a level of toxin activity beyond surface receptors.

3.5 Endosomal Acidification Studies Using FITC-EGF:

Since some of the mutants appeared to possess normal levels of toxin receptors, I wished to examine the ability of the mutants to acidify their endosomes. In RME, the ligand associates with its receptor on the cell surface. After the formation of the ligand-receptor complex, the ligand is internalized into an endocytic vesicle. Once inside the vesicle, the pH of the compartment drops, because of the activity of a membrane-bound ATP-driven proton pump, and the ligand-receptor complex dissociates. The ligand is then further internalized, and in some cases, the receptor returns to the cell surface. Many hormones gain access to the cell via RME. However, many toxins also utilize this pathway to reach their intracellular target. Toxins utilize the pH difference which forms across the endosomal membrane to drive the enzymatic α-subunit through the membrane and into the cytoplasm of the cell. For example, diphtheria toxin may enter the target cell cytoplasm by RME. Diphtheria toxin-resistant CHO cells have been isolated which are unable to lower the endosomal pH (Moehring *et al.*, 1983). To examine the ability of the VT-resistant cells to acidify their endosome, I adapted a procedure first described by Ohkuma and

Poole (1978). The procedure makes use of the finding that the fluorescence emission wavelength of the FITC molecule is dependent on pH. In the Ohkuma and Poole procedure, FITC labelled dextran was used as a probe for endosomal pH. However, dextran is a relatively insensitive reporter molecule because much of the surface-associated dextran does not enter the cell. Consequently, I used FITC-labelled EGF which is known to be internalized by RME. Prior to using FITC-labelled EGF as a probe for endosomal pH, I performed several control experiments to evaluate the binding characteristics of FITC-labelled EGF. In the first experiment I examined the ability of underivatized EGF to compete for FITC-EGF binding to cells. In addition, the A 431 cell line was used in the control experiments because this cell line has a large number of EGF receptors on its surface (Krupp *et al.*, 1982). I demonstrated that the FITC-EGF did associate with the A 431 cells, and underivatized EGF was able to compete for the binding of the FITC-EGF (Plate 7). To demonstrate this in a more quantitative manner, I grew monolayers of A 431 cells in 24-well plates and performed a radioreceptor binding experiment with ^{125}I-labelled FITC-EGF. I also performed the radioreceptor binding experiment with Vero and HeLa 229 cells to quantitate the number of EGF receptors in these cell lines (Figure 3). As expected, the A 431 cells possessed much greater levels of EGF receptors than either of the other two cell lines.

Plate 7: Binding of FITC-EGF to A 431 Cell Monolayers. A 431 cell monolayers were grown on glass slides for 24 hours at 37°C in a humidified 5% CO_2 atmosphere.

Panel A: Unlabelled EGF was bound to the cells for 15 minutes on ice prior to the addition of FITC-labelled EGF. FITC-EGF was then added to the cells and allowed to bind for a further 45 minutes. The slides were washed, fixed in -20°C acetone, air-dried and mounted in PBS/glycerol. The cells were viewed with a fluorescence microscope at the wavelength of 493.5 nm.

Panel B: FITC-EGF was bound to A 431 cells for 45 minutes on ice in the absence of unlabelled EGF. At the end of the incubation period the cell monolayers were prepared for fluorescence microscopy as described above.

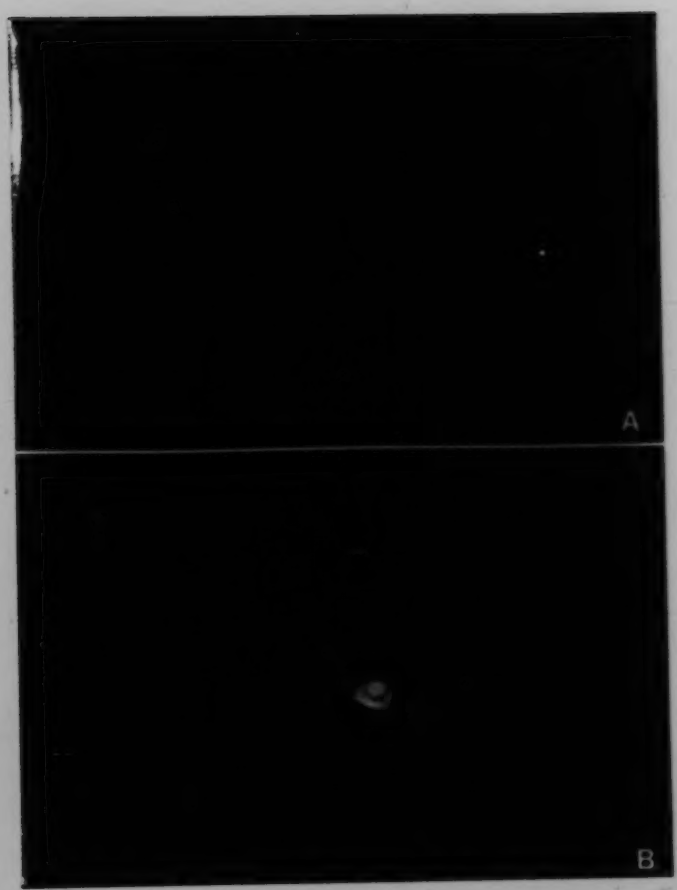
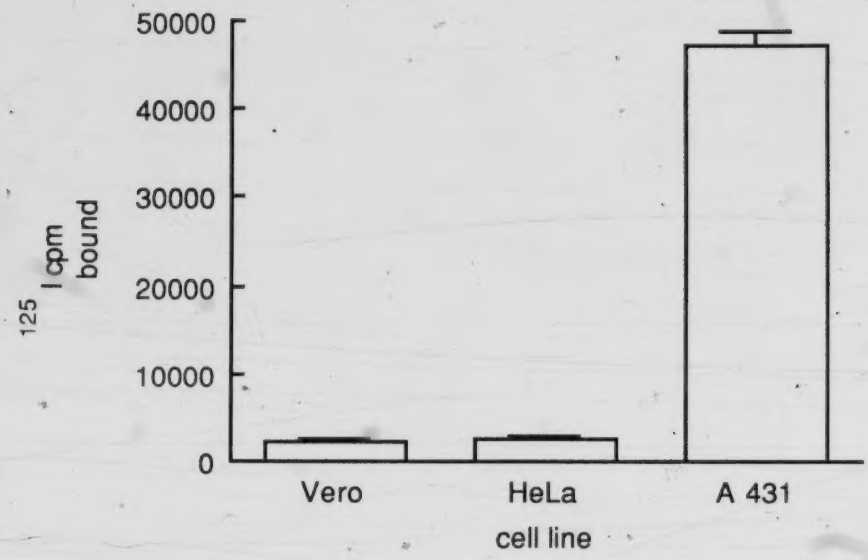

A
B

Figure 3: Specific Binding of ^{125}I-Labelled FITC-EGF to Vero, HeLa, and A 431 Cell Lines. The specific binding represents the difference between counts bound in the presence and absence of unlabelled EGF. The error bars represent the standard deviation of the mean of triplicate determinations.


50000
40000
30000
20000
10000
0
^{125}I cpm bound
Vero
HeLa
A 431
cell line

Nonetheless, specific binding of FITC-EGF was demonstrated to HeLa and Vero cells.

The FITC molecule is sensitive to photobleaching when exposed to light for extended periods of time. To control for this effect, I performed further control experiments with tissue culture cells which were unable to internalize the FITC-labelled EGF. In addition, the experiment was performed in the presence of monensin to block acidification of the endosomes (Table 10). In the first of the control experiments I bound FITC-EGF to A 431 cells on ice at 4°C for 30 minutes. I then placed the cell monolayer in -20°C acetone for 10 minutes to fix the cells and then placed the coverslip containing the acetone-fixed monolayer into the coverslip holder in the spectrofluorometer. In this experiment the intensity of the fluorescence at 519 nm remained essentially constant or even increased slightly during the incubation period (Table 10 and Plate 8). From this result I concluded that the FITC label was stable to photobleaching for at least 30 minutes in the light beam of the spectrofluorometer (Plate 8). Further, the CHO cell mutants which displayed a decreased level of endosome acidification over the parental cells (Moehring and Moehring, 1983) and HeLa cells which were exposed to monensin also displayed a reduced rate of decline in fluorescence intensity (Table 10). However, none of the toxin-resistant clones appeared to have significant defects in endosome acidification. In fact, HeLa cm:2 and HeLa cm:3 appeared to have an increased rate of ligand internalization

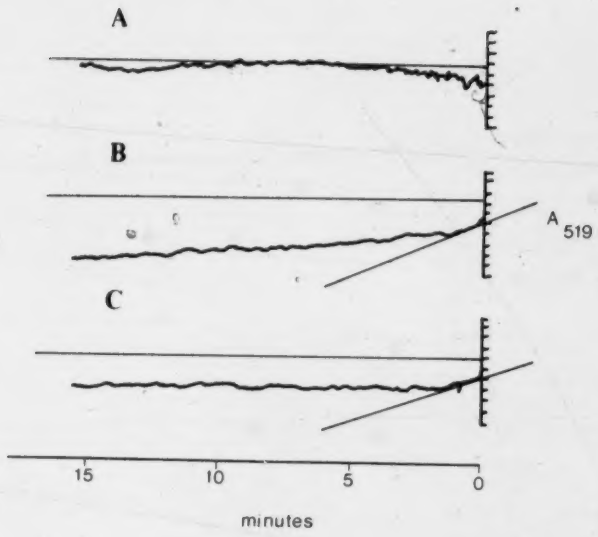
Table 10

Results of the FITC-EGF Endosomal Acidification Assay

cell type	Experimental Conditions	δ[- slope[a]] = A_{519}/min ± SEM[b]
HeLa 229	--	0.444 ± 0.06
HeLa cm:1	--	0.484 ± 0.16
HeLa cm:2	--	0.540 ± 0.04[c]
HeLa cm:3	--	0.680 ± 0.10[c]
Vero	--	0.256 ± 0.04
Vero cm:3	--	0.200 ± 0.05
Vero cm:17	--	0.180 ± 0.02[d]
A431 cell	acetone fixed	0.000
HeLa 229	15 mM monensin	0.187 ± 0.07
	25 mM monensin	0.240 ± 0.10
	50 mM monensin	0.063 ± 0
CHO Parental	--	0.291 ± 0.05
CHO (RPE 44)[e]	--	0.150 ± 0.03

[a]quenching of fluorescence is expressed as the absolute value of the decreasing initial quenching rates monitered at 519 nm. The initial rate of the decrease in absorbance was calculated from the slopes of the lines displayed in Plate 8.
[b]SEM is the standard error of the mean (n=3).
[c]significance $p<0.1$ when compared to parental strain. p value calculated by Student's t-Test
[d]significane $p<0.05$ when compared to parental strain. p value calculated by Student's t-Test.
[e]Endosomal acidification deficient strain obtained from Dr. J. Moehring, Burlington, Vermont, USA.

Plate 8: Emission Quenching Curves Obtained During Endosomal Acidification Assay Using FITC-EGF. (A) Acetone fixed A 431 Cells. Control used to determine stability of FITC probe to exposure of the incident excitation light beam (B) Sample data obtained with HeLa 229 parental cells. (C) Sample data obtained with parental Vero cells. The initial quenching rate was calculated from the slopes of the lines shown in traces B and C.


A
B
C
A_{519}
15
10
5
0
minutes

and endosome acidification ($p<0.05$) in comparison to the parental HeLa 229 cells (Table 10).

3.6 Determination of the Minimum Inhibitory Concentration (MIC) of Mitomycin C on growth of *Escherichia coli* O157:H⁻ and O26:H11:

In order to further characterize the VT-resistant cells and confirm the results of the Gb_3 analysis, I wished to perform radioreceptor binding assays using purified ^{125}I-labelled VT. Therefore, my next series of experiments were directed at establishing a VT purification procedure. To begin with, I performed experiments to determine the conditions necessary for production of the maximum amount of toxin in the bacterial cultures. From this study I found that mitomycin C, at a concentration of 0.5 µg/mL, effectively inhibited the growth of both bacterial strains and I found that a concentration of 0.2 µg/mL of mitomycin C was sub-inhibitory to both *E. coli* strains. Therefore, I chose to use mitomycin C at a concentration of 0.2 µg/mL, as it was the maximum concentration of mitomycin C which did not appear to appreciably inhibit bacterial growth.

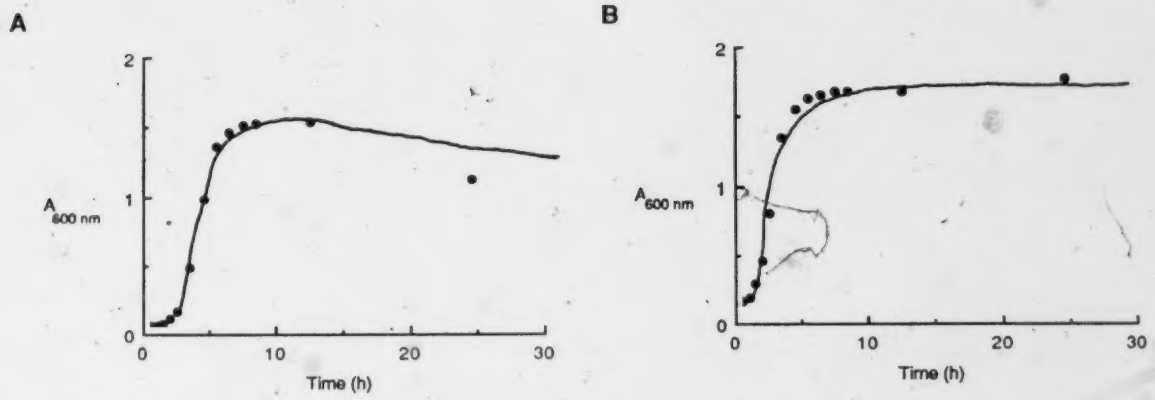
When I inoculated test cultures with 0.2 µg/mL mitomycin C and compared the toxin titre to control cultures which did not contain mitomycin C, I found that, in the absence of mitomycin C the toxin CD_{50} was 10^3, in cultures containing mitomycin C the CD_{50} was approximately 10^6, a difference of 1000 times. This experiment was performed on

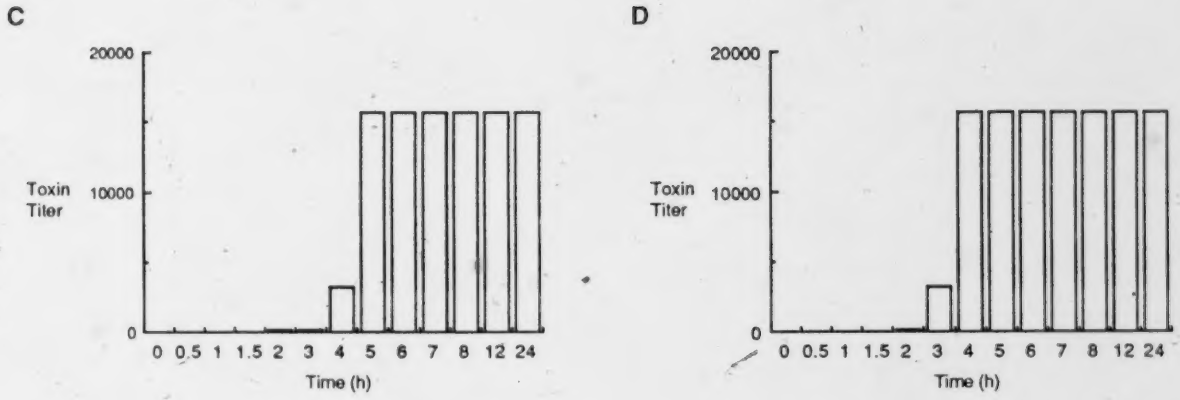
two separate occasions, and the difference between the toxin CD_{50} between control and mitomycin C-treated cultures ranged from 100-1000 fold. A control employing 0.2 µg/mL of mitomycin C alone in Vero cell monolayers, did not induce changes which resembled cytotoxicity caused by VT.

3.7 Relationship Between Growth Phase and Toxin Production in *E. coli* O157:H$^-$ and *E. coli* O26:H11:

To determine an optimal incubation period which yielded maximum toxin production, I determined bacterial growth curves and concurrently measured toxin titres on aliquots removed from the parent cultures (Figure 4). I found that, in the presence of 0.2 µg/mL of mitomycin C, cultures of *E. coli* O157:H$^-$ and *E. coli* O26:H11 reached the maximum limits of their growth in approximately 6 hours at 37°C in a shaking incubator. The CD_{50} titre of toxin produced by each of the two serotypes also reached maximum levels (CD_{50}=15,600) at this time. At 24 hours incubation the A_{600} of *E. coli* O26:H11 had decreased compared to the A_{600} of the culture at 12 hours incubation. This could be due to lysis of the bacteria because the bacteriophage harbouring the verocytotoxin genes may be entering the lytic phase of growth.

Figure 4: Growth curves of *Escherichia coli* O26:H11 (Panel A) and *Escherichia coli* O157:H$^-$ (Panel B) in TSB Containing 0.2 µg/mL of mitomycin C. At each time interval the absorbance of an aliquot of the culture was measured at A 600 nmin a Gilford Model 250 Spectrophotometer using a cuvette with a 1 cm light path. The titre of toxin produced by *E. coli* O157:H$^-$ and *E. coli* O26:H11 is presented in panels C (O157:H$^-$) and D (O26:H11). Each bar represents the mean of two determinations at the indicated time intervals. Aliquots were titered on Vero cell monolayers as described in the Materials and Methods section. The toxin titre represents the reciprocal of the dilution which caused death of half of the cells present in the tissue culture well.


A
$A_{600\ nm}$
2
1
0
0
10
20
30
Time (h)
B
$A_{600\ nm}$
2
1
0
0
10
20
30
Time (h)


C
Toxin Titer
20000
10000
0
0 0.5 1 1.5 2 3 4 5 6 7 8 12 24
Time (h)
D
Toxin Titer
20000
10000
0
0 0.5 1 1.5 2 3 4 5 6 7 8 12 24
Time (h)

3.8 Characterization of the SLT-I and SLT-II Monoclonal Antibodies Reaction with the Bacterial Strains *E. coli* O157:H$^-$ and O26:H11:

Any VT which is not neutralized by anti-Shiga or anti-VT-1 is termed VT-2. To determine if the VT-2 I was using would react with an anti-SLT-II monoclonal antibody which I had obtained from Dr. Nancy Strockbine (CDC, Atlanta, Georgia, USA), an ELISA using the bacterial strains *E. coli* O157:H$^-$ and *E. coli* O26:H11 was performed. This monoclonal antibody had been raised against a strain of *E. coli* O157:H7. As well as using the anti-SLT-II monoclonal antibody, I also used an anti-SLT I antibody which I had obtained from the ATCC Culture Collection. The SLT I monoclonal antibody has been reported to react with an epitope in the β-subunit of VT-1 (SLT-I) and neutralizes Shiga toxin activities from *Shigella dysenteriae* Type I and *S. flexneri* and Shiga-like toxin activities from *E. coli*, *Vibrio cholerae*, *V. parahemolyticus* and *Salmonella typhimurium* (Strockbine *et al.*, 1985).

To confirm that the bacterial strains (*E. coli* O157:H$^-$ and *E. coli* O26:H11) used in the study produced VT-1 or VT-2, I performed a verocytotoxicity assay with filter-sterilized, polymyxin B extracts from each strain. The cytotoxicity assay demonstrated that both strains produced toxins which were lethal for Vero cells. The CD_{50} (the endpoint of the toxin titration defined as the reciprocal of the dilution of

the extracted toxin required to kill 50% of the cell monolayer during the 48 hour incubation period) of the broth extract prepared from both cultures was 64.

The ELISA procedure revealed that *E. coli* O26:H11 produced VT-1. In contrast, VT-1 production was not detected in culture fluid from *E. coli* O157:H$^-$ (see Table 11). The monoclonal anti-VT-2 I used did not react with the toxin produced by *E. coli* O26:H11, nor did it react with toxin from *E. coli* O157:H$^-$. I therefore concluded that *E. coli* strain O26:H11 produced VT-1 and that *E. coli* O157:H$^-$ did not produce VT-1 but produced a verocytotoxic factor which failed to react with the SLT-II monoclonal antibody obtained from Dr. Strockbine, but had been designated VT-2 by Scotland *et al.* (1985).

3.9 Results of Purification Protocol of VT-2:

In the first step of the purification procedure, I concentrated the toxin from polymyxin-treated bacterial cell pellets by ammonium sulfate precipitation. The polymyxin treatment was required to solubilize the outer membranes of the *E. coli* and release toxin from the periplasmic space.

At this stage the crude toxin extract was noticeably yellow in colour. However, the yellow coloured material was removed when the extract was passed through a hydroxylapatite column as described in the materials and methods section. Fractions which were eluted from the hydroxylapatite column (Figure 5) which contained high levels of protein and

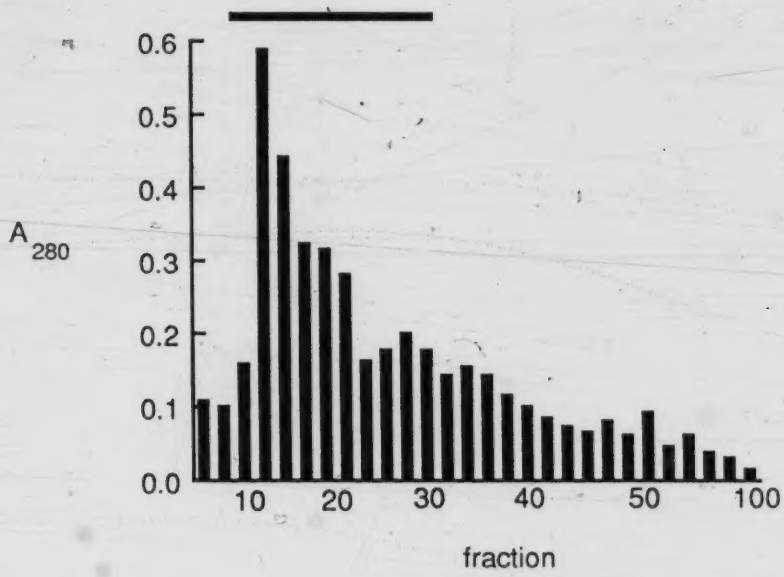
Table 11

Characterization of Bacterial Strains
Used in the Study

Bacterial strain	Toxin[a] Production	ELISA Results[b] Anti-VT-1	Anti-VT-2
E. coli O157:H⁻[c]	(+)	0	0
E. coli O26:H11	(+)	10^4	10^1

[a]Toxin Production was determined with the Verocytoxicity assay.
[b]ELISA titre=reciprocal of the dilution of the monoclonal antisera which gives an O.D. of 0.050 when the least diluted antisera equals an O. D. of 2.000
[c]Prototypic VT-2 producer (Scotland *et al.*,1985).

Figure 5: Elution Profile of Hydroxylapatite Column. The column was equilibrated and the VT-2 Polymyxin extract was applied in 0.01 M potassium phosphate buffer. Bound material was eluted with 0.05 M potassium phosphate. The absorbance of each fraction was measured in a Gilford Model 250 Spectrophotometer at a wavelength of 280 nm using a cuvette with a 1 cm light path. The A_{280} of each fraction is represented as a filled bar. The fractions (8-35) which contained verocytoxicity (horizontal bar) were pooled.


A280
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10
20
30
40
50
100
fraction

verocytoxic activity were pooled and subjected to IEF using a Bio-Rad Rotofor™ liquid phase IEF apparatus. In this procedure the toxin migrates in an electrical field until its pI is reached. The pI of a protein is the point at which its net charge equals zero. At this point, the proteins stop migrating and concentrate into tight bands. I used a form of preparative IEF which utilizes a liquid matrix rather than methods which use solid matrixes such as acrylamide or sephadex gels. The liquid-phase IEF procedure has several distinct advantages over those forms employing solid matrixes. The first advantage is that a rapid and efficient vacuum system is used to remove the focussed samples from the liquid matrix, whereas, in the solid phase system, the samples must be removed by scooping the gel out of the gel frame, or by cutting the acrylamide. The second advantage is that proteins separated by the liquid phase preparative IEF method do not need to be extracted from the gel matrix. The pH gradient which was generated in the Rotofor apparatus is shown in Figure 6. The IEF fractions which contained cytotoxic activity and protein bands corresponding to the VT α and β subunits (Plate 9) were pooled for further purification.

The next step in the purification procedure was Blue Sepharose affinity chromatography. This procedure takes advantage of structural similarities between nucleotides and the dye Cibacon Blue to purify nucleotide-binding proteins. It has been used successfully in the purification of Shiga

Figure 6: pH Gradient Generated by the Rotofor™ Isoelectric Focussing Apparatus During the VT-2 Purification Procedure. The bars represent the pH value of each fraction generated by the liquid phase isoelectric focussing apparatus using Biolyte brand ampholytes with a pH range of 6-8. The anode solution used was 0.1 M NaOH and the cathode solution was 0.1 M H_3PO_4. Isoelectric Focussing was performed using a constant power of 12 watts for 5 hours. Each fraction was analyzed for Vero cytotoxicity on Vero cell monolayers. The fractions which possessed the highest titre of cytotoxicity were pooled. These fractions are indicated by a heavy bar at the top of the graph.

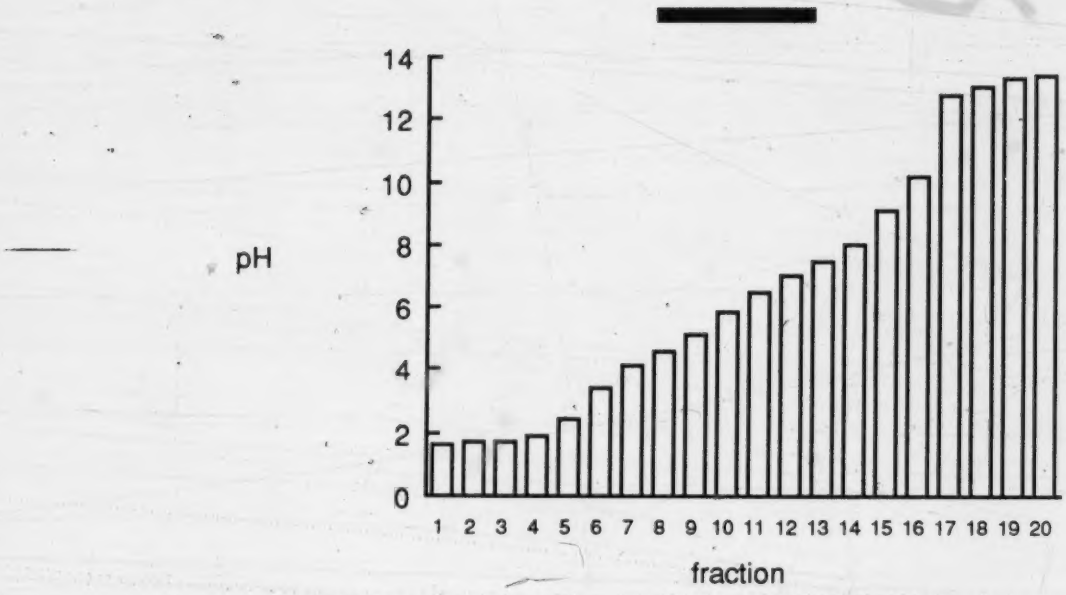
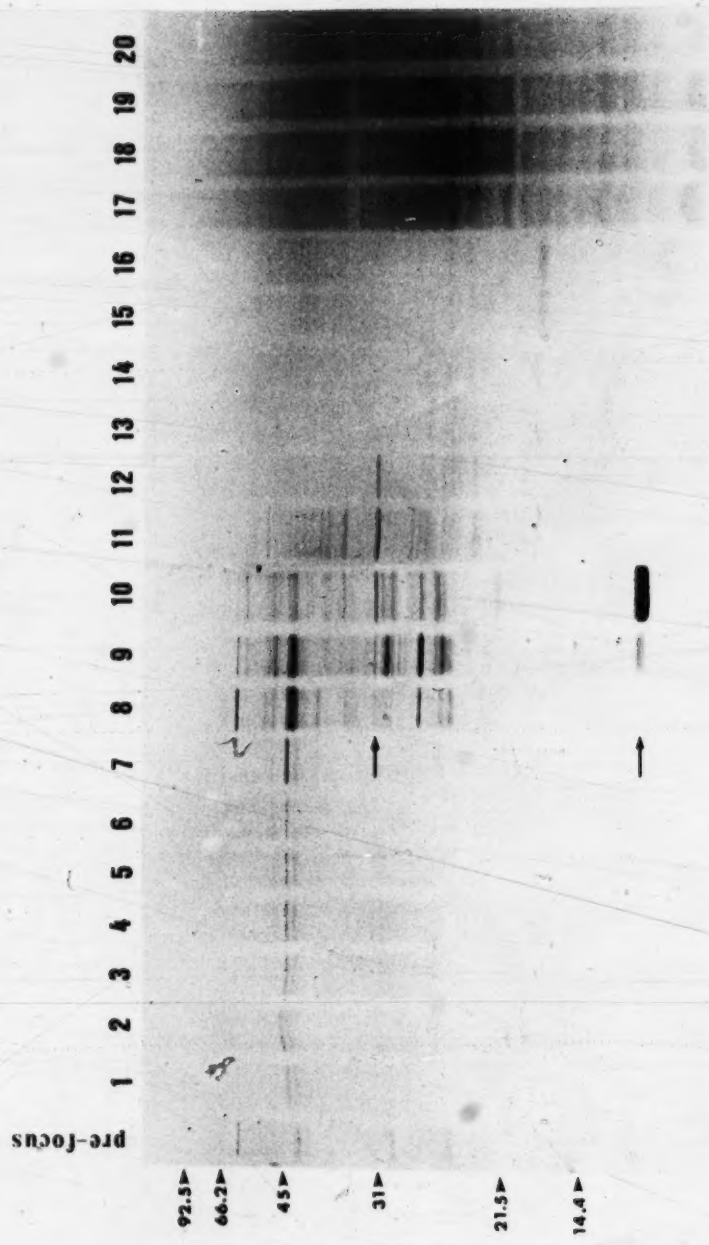

pH
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
fraction

Plate 9: SDS-PAGE analysis of the Isoelectric Focussing Fractions of VT-2. SDS-PAGE was performed using the discontinuous gel buffer system described by Laemli and a 16% seperating gel. Samples were exposed to 2-mercaptoethanol prior to electrophoresis. The IEF procedure was performed as described in the Material and Methods Section 2.19. The lane marked pre-focus shows the proteins present in the starting material. The next lanes marked 1-20 are the 20 fractions which were generated by the focussing apparatus. The upper arrow indicates a protein band with a molecular weight of approximately 31 K. The lower arrow indicates a protein band with a molecular weight of approximately 6 K. These two bands correlate to the weights of the α and β subunits of VT-2 (Head *et al.*, 1988). The positions of the standards are indicated to the left of the photograph.


pre-focus
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
92.5
66.2
45
31
21.5
14.4

toxin (O'Brien *et al.*, 1983) and VT-1 (Petric *et al.*, 1987) because the enzymatic portion of the toxins bind to adenine. However, VT-2 seemed to have a lower affinity for the matrix. I found that the cytotoxic activity recovered from the column was as great in the flow-through fractions (Table 12) as it was in the fractions eluted with 50 mM sodium phosphate.

Results of the purification procedure for VT-2 are summerized in Table 12. Fractions obtained at each stage of the process are shown in Plate 10. The overall purification procedure resulted in a recovery of 0.0002% of the starting cytotoxin activity from 50 mL of polymyxin extracted *E. coli* O157:H$^-$ (harvested from a 12 L culture). It was obvious that the purification procedure yielded a very small amount of the original toxin activity.

3.10 Results of Purification of VT-1:

The first steps in the purification of VT-1 were similar to those employed in the purification of VT-2 with the exception that the polymyxin extracts were not applied to a hydroxylapaptite column to decrease sample preparation time. It was felt that the hydroxylapaptite procedure could be omitted because SDS-PAGE analysis suggested that the IEF procedure might be able to perform the task of hydroxylapaptite. In addition, since I had obtained a VT-1 specific monoclonal antibody, I decided to omit the Blue Sepharose chromatography step and proceed directly from IEF

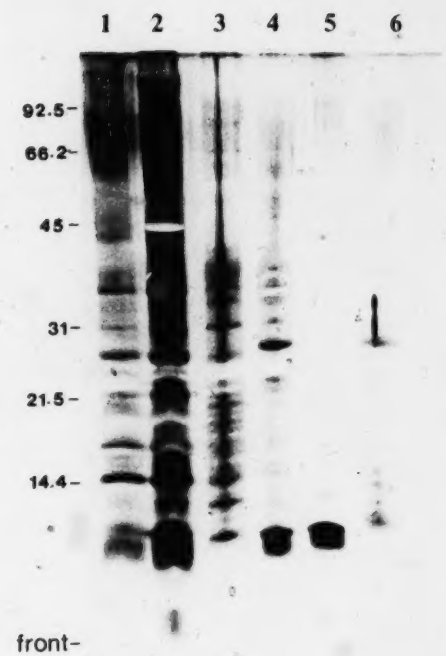
Table 12

Summary of VT-2 Purification Procedure

	Volume (mL)	Protein[a]	Activity[b] (CD_{50})	Sp Activity (CD_{50}/mg protein)	%Recovery[c]
$(NH_4)_2SO_4$ ppt. polymyxin extracted	50	200	10^5	500	100
Hydroxyapatite eluate	50	150	10^4	660	99
IEF Fraction	4	ND[d]	10^5	ND[d]	--
Blue Sepharose (flow through)	1	0.5	100	200	0.0002
Blue Sepharose (eluate)	1	0.5	100	200	0.0002

[a] Protein = mg/total volume determined by the Lowry procedure (Appendix 2).
[b]Reciprocal of dilution resulting in death of 50% of the cells per monolayer.
[c]Expressed as $100[(CD_{50} \times mL/fraction)/(50 \times 1x10^5)]$.
[d]Unable to determine due to interferance in assay from ampholyte.

Plate 10: Silver Stained Samples Obtained at Each Step of the Purification Procedure of VT-2 and analyzed on a 16% discontinuous SDS-PAGE gel. The positions of the protein standards are indicated by the molecular weights (x 10^3) on the left side of the photograph. Lane 1 is a sample of the cell-free culture medium. Lane 2 is the sample which was extracted with polymyxin B, and clarified by ultracentrifugation, and concentrated by 40% ammonium sulfate precipitation. Lane 3 is a sample of the material eluted from the hydroxylapatite column. Lane 4 is the sample after it has been subjected to preparative isoelectric focussing using the liquid phase Rotofor™. Lane 5 is a sample of material elut . from the Blue Sepharose as described in the Materials and Method Section 2.19 and Lane 6 shows material which was not absorbed to Blue Sepharose.


1
2
3
4
5
6
92.5-
66.2-
45-
31-
21.5-
14.4-
front-

to immunoaffinity chromatography using the VT-1 monoclonal antibody.

SDS-PAGE analysis of VT-1 fractions obtained during the various purification steps are presented in Plate 9. It was apparent from the photographs presented in plate 9 that the IEF procedure resulted in the loss of protein bands in the 66 to 93 and 22 to 31 Kdal range which were present in the polymyxin extracts (Plate 11, compare Lanes 2 and 3). The pH gradient generated by the liquid phase IEF method is shown in Figure 7. However, after the toxin extract was subjected to immunoaffinity chromatography using the monoclonal antibody (Plate 11), no protein bands were detected on the silver stained gel (Plate 11, Lane 4). Nonetheless, the immunoaffinity purified toxin extract did produce cytotoxic effects in Vero cells (see Table 13). Therefore, the concentration of the toxin in the immunoaffinity purified sample was probably below the detection level of the silver stain procedure.

The results of the various steps in the purification of VT-1 are presented in Table 13. The overall recovery of cytotoxic activity by the VT-1 purification procedure resulted in approximately 0.0001% recovery of cytotoxic activity.

Plate 11: Silver Stained Samples Obtained at Each Step of the Purification Procedure of VT-1 and analyzed on a 16% discontinuous SDS-PAGE gel. The positions of the standards are indicated by their molecular weights (x 10^3) on the left side of the gel. Lane 1 is a sample of the cell-free culture supernatant solution. Lane 2 is a sample from the bacterial cell pellet which was extracted with polymyxin B and concentrated by ammonium sulfate precipitation. Lane 3 is the sample which has been subjected to preparative liquid phase isoelectric focussing in the Rototfor™ Apparatus. Lane 4 is the sample after immunoaffinity chromatography.

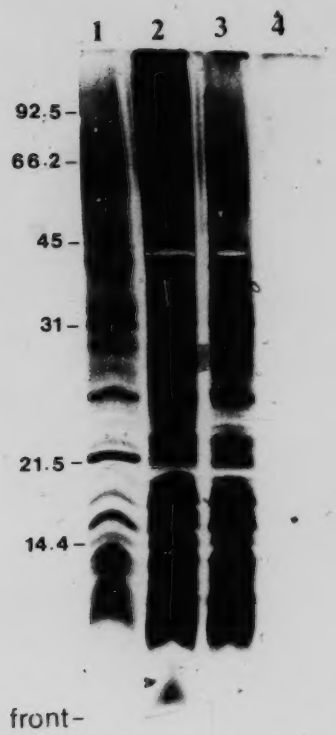

1
2
3
4
92.5
66.2
45
31
21.5
14.4
front

Figure 7: pH Gradient Generated by the Rotofor™ Isoelectric Focussing Apparatus During the VT-1 Purification Procedure. The bars represent the pH value of each fraction generated by the liquid phase isoelectric focussing apparatus using Biolyte brand ampholytes (pH range 6-8). IEF was performed as described in the legend to Figure 6. The fractions which possessed the greatest amount of cytotoxicity were pooled. These fractions are indicated by a heavy bar at the top of the graph.

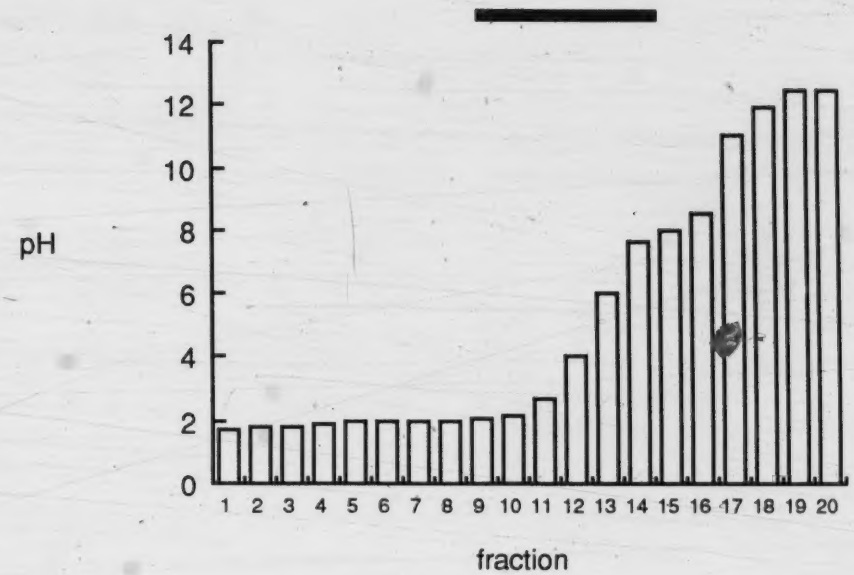

14
12
10
8
6
4
2
0
pH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
fraction

Table 13

Summary of VT-1 Toxin Purification Procedure

	Volume (mL)	Protein[a] (mg/mL)	Activity[b] (CD_{50})	Sp Activity (CD_{50}/mg protein)*	%Recovery[c]
cell pellet	30	90	10^6	11000	100
IEF Fraction	16.5	ND[d]	10^9	ND[d]	ND[d]
Anti-VT-1 bound Fraction	0.3	0.024	10^2	333	0.0001

[a] Protein = mg/total volume. Protein determined by the Lowry procedure (Appendix 2)
[b]Reciprocal of dilution resulting in death of 50% of the cells per monolayer
[c]Expressed as $100[(CD_{50} \times mL/fraction)/(50 \times 1x10^5)]$
[d]Unable to determine due to interferance in assay from ampholyte.

3.11 Radioreceptor Assays Performed With Purified VT-1 and VT-2:

Although I was unable to obtain purified toxin as demonstrated by two bands on SDS-PAGE, I attempted to perform radioreceptor binding assays with the material I was able to prepare. Perhaps, not surprisingly, I was unable to demonstrate specific binding of the ^{125}I-labelled VT preparations to parental HeLa or Vero cells. There were at least two possible explanations for this result. First, it was conceivable that the concentration of purified, unlabelled VT was too low to compete for the binding of ^{125}I-labelled VT to the HeLa or Vero cell membranes. This problem would have been compounded if the purified VT samples contained contaminating proteins which became labelled with iodine, but were unable to bind to VT receptors. Second, it was possible that the iodination procedure had destroyed VT binding activity in the same way that iodination destroys the binding of pertussis toxin (Armstrong and Peppler, 1987). However, the second possiblity seemed unlikely because Lingwood *et al.* (1987) have been able to produce iodinated VT-I which retains its binding ability to Gb_3. Moreover, I was able to iodinate the VT-1 which I had produced and demonstrated specific binding of ^{125}I-VT-1 to Gb_3 in a radioreceptor tube binding assay (Table 14). This demonstrated that even though the material which I produced may not have been purified to homogenity, it did retain binding acivity in the iodinated form.

Table 14

Binding of VT-1 and VT-2 to GM_1 and Gb_3 Coated Glass Culture Tubes

Toxin	Glycolipid coating tube	Average cpm Bound ± SEM[a]
VT-1[b]	GM_1[d]	2104 ± 356.81
	Gb_3	5567 ± 918.50
VT-2[c]	GM_1	691 ± 175.38
	Gb_3	1793 ± 127.54

[a]SEM is the standard error of the mean (n=3).
[b]VT-1 purified using my purification procedure.
[c]VT-2 obtained from S. Head (University of Toronto).
[d]Ganglioside GM_1 was used as a control. It does not contain the terminal Gal(α1-4)Gal disaccharide which is thought to be the receptor for VT-1 and VT-2.

4. Discussion

The objective of this investigation was to investigate the mammalian tissue receptor for VT-2. My approach was to isolate HeLa and Vero cell clones which were resistant to VT-2 and compare these to the parental cell lines. It was reasoned that by obtaining clones with increased resistance to the toxin I would obtain some clones with reduced numbers of receptors. Not only would this allow me to identify the cell surface components which were absent in the toxin-resistant clones but, more importantly, the receptor-defective cells could be used in reconstitution experiments to restore receptor activity to the resistant cells.

I isolated seven VT-2 resistant HeLa clones and six Vero clones by spontaneous selection with a crude VT-2 toxin extract. From each of the isolated clones, I chose the most resistant based on their initial growth characteristics in the presence of VT-2 (Tables 4, 5 and 9) (HeLa cm:1, HeLa cm:2 and Vero cm:3) for further characterization. I also decided to examine two other clones (HeLa cm:3 and Vero cm:17) not because they were the most resistant, but because they displayed short-term resistance to the toxin. This was demonstrated in growth experiments (Figure 1 and 2). In contrast, during the long-term exposure to the toxin in the plating efficiency experiments, HeLa cm:3 and Vero cm:17 did not remain viable (Table 6). Also, these cells exhibited a

decreased growth rate in culture when not in the presence of toxin (Table 4 and 5). I felt that this characteristic may be compatible with a defect in the entry mechanism of important growth factors into these cells through a process such as receptor-mediated endocytosis.

I feel that I did not isolate mutant cell lines as such from the parental cell line, but rather isolated a toxin-resistant sub-population of cells which were already established within the toxin-sensitive population. My reasoning is that spontaneous mutations occur in haploid organisms (bacteria) at a frequency of 1 in 10^7 to 1 in 10^8. In diploid cells (mammalian cells) this rate would be even lower because the resistant phenotype would only appear in cells which are homozygous for the mutated gene. However, I was able to obtain at least 13 isolates from approximately 3 million cells which is equivalent to an isolation rate of 4 per 10^6 and is, therefore, too high to be explained by a spontaneous mutation. Therefore, the VT-toxin-resistant cells must have been present before the selective pressure was applied. These may have arisen as spontaneous mutations which occured in the past and were maintained in the population, or the resistant cells may have been at a level of differentiation which was different from the majority of the population. Whatever the origin of the resistant cells, the nature of the differences between them and the remainder of the toxin-sensitive cells may have been such that they have been able to compete for survival within the population.

Recent data suggests that VT-1 and VT-2 are structurally and functionally related (Jackson *et al.*, 1987; Hovde *et al.*, 1988), but their structural similarities do not extend to immunological cross-reactivity (Scotland *et al.*, 1985; Karmali *et al.*, 1986; Strockbine *et al.*, 1986). I was also interested in determining if VT-2 resistant clones were resistant to VT-1. In order to determine this however, I had to confirm that the *E. coli* O157:H$^-$ (E32511) strain, the producer of VT-2, did not produce VT-1 also. This was accomplished by performing an ELISA with VT-1 and VT-2 monoclonal antibodies. However, during the ELSIA trials I discovered that the O157:H$^-$ strain I had obtained from Scotland and Rowe produced a toxin which did not crossreact with anti VT-2 antibodies which were originally described by Strockbine et al. (1988). This observation suggested that immunogenic diversity among the verocytotoxins and the shiga-like-toxins may be greater than was previously known. The situation with the SLTs and VTs may be similar to that of the cholera and heat-labile family of toxins expressed by enterotoxingenic *E. coli*. The cholera toxin family is now known to contain at least 6 antigenically unrelated members (Finkelstein *et al.*, 1987). Nonetheless, all of the members of the cholera toxin family are functionally related.

When I exposed the VT-2 resistant clones to VT-1, I found that clones which displayed resistance to VT-2 also displayed a degree of resistance to VT-1 (Table 6 and 7), which implied that the mechanism of resistance to the two

toxins was similar. However, I did obtain results which suggested that HeLa cm:5 appeared to be much more resistant to VT-1 than VT-2 (Table 6 and 7), having a plating efficiency of 55.8% when exposed to VT-1, but only 1.4% in the presence of VT-2. In contrast, Vero cm:3, appeared to be more resistant to VT-2 (plating efficiency 59.3%) than to VT-1 (plating efficiency 0% [Table 6 and 7]). This was more of an expected result because VT-2 was the selection agent used to isolate the clones.

From these observations I concluded that the mechanism of resistance must be very similar between the toxins, but that there may be slight differences in the way that the toxins interact with the cells. To try to elucidate the differences and similarities I further characterized the clones.

At first I considered that the mechanisms of resistance might have been due to a membrane-bound enzyme which could degrade the toxin before it had a chance to enter the tissue culture cells. However, this seemed unlikely because the selection procedure was performed in the presence of protease inhibitors in FBS. Nevertheless, such a mechanism cannot be completely ruled out in toxin-resistant clones which appeared to have normal levels of receptors and a functional RME pathway.

The differences in the protein patterns observed when the VT-resistant clones were examined by SDS-PAGE further demonstrate that the toxin-resistant cells are unlike the

parental cells. These differences may have been unrelated to the mechanism of toxin resistance. Alternatively, the protein profiles may be related to differences in glycolipid content of the membranes or functional receptors may be glycolipid-protein complexes. Lingwood *et al.* (1987) has proposed that the mammalian cell VT-1 receptor is the neutral glycolipid Gb_3. Since I did not observe any consistent differences in the protein profiles of the HeLa and Vero cells, I decided to investigate if my clones contained reduced levels of Gb_3. I found that the most resistant HeLa cell clones (HeLa cm:1, HeLa cm:2) had Gb_3 levels which were approximately 50% that of the parental cells, and Vero cm:3 had a Gb_3 level which was 80% that of the parental cells. Since their initial work, Waddell *et al.* (1988) were able to reconstitute VT-2 sensitivity into mutant Daudi cells with liposomes containing Gb_3. Therefore, the receptor for VT-2 may also be Gb_3. The observation that VT-2 resistant clones have reduced levels of Gb_3 is consistent with the finding of Waddell *et al.*, (1988). Nonetheless, Waddell *et al.* has been unable to fully reconstitute toxin sensitivity in the Gb_3-treated, Daudi cells (personal communication). This supports my proposal that the receptor for VT-2 may be a Gb_3-containing complex. I also noted that other glycolipids, which were metabolically labelled with ^{14}C-serine, also appeared to be decreased in concentration in comparison to the parental cells. The importance of these glycolipids in the binding of VT-2, however, must still be determined. This could be

accomplished through binding of ^{125}I-labelled VT-2 to these glycolipids on the thin-layer chromatography plates.

To confirm the mechanism of resistance in the clones which expressed lower levels of Gb_3, I wished to perform a radioreceptor binding assay using iodinated, purified toxin. This would also have allowed me to compare the affinity of receptors for VT-1 and VT-2 in toxin-sensitive and toxin-resistant cell lines. It is also possible that differences in the sensitivity of some of the toxin-resistant clones may be related to different affinities of VT-1 and VT-2 for Gb_3. However, my attempts at obtaining pure VT-1 and VT-2 met with only limited success. Nevertheless, the purification studies have provided additional insights into the biochemical nature of these toxins.

One of the steps in the purification of VT-1 and VT-2 was preparative, liquid-phase isoelectric focussing. When the liquid matrix was fractionated at the end of the isoelectric focussing procedure, I noticed that the α and β subunits of the toxin did not co-purify in the same fractions (Plate 10). This suggested that the association of the α and β subunits may not be very strong *in vitro*, the toxin subunits may not have a fixed stoichiometric relationship. This may explain why a consensus has not been reached on the exact α:β stoichiometry for the VTs. Moreover, the dissassociation observed during the IEF may explain why my recovery of toxin activity from the purification procedure was so low. Although the α and β subunits may not be tightly

associated, there may be an optimal ratio which is necessary for full activity. The IEF procedure may have shifted the α:β ratio such that the toxin was less active in the Vero cell assay system. This may have resulted if the preparations contained an excess of β subunits which could compete for active-toxin binding to receptors.

The next step I used in the purification of VT-2 was an affinity chromatography procedure. This procedure exploited the nucleotide-binding activity of the α-subunit and the resemblance of the dye Cibacron Blue to nucleotides. Although this step appeared to be useful for the purification of Shiga toxin and VT-1 (O'Brien *et al.* 1984; Petric *et al.* 1987), in the purification of VT-2, I found that much of the toxin passed through the column without binding and was recovered in the wash fractions (Table 12). This observation also suggested that there may be functional differences between VT-1 and VT-2 which may also be related to the immunological differences and activity differences detected in some of the VT-resistant HeLa and Vero cell clones.

Although I did not obtain a purified toxin, I determined that the partially purified VT-1 which was obtained would bind to Gb_3 in a radioreceptor type assay (Table 14). In contrast, neither VT-1 nor VT-2 (obtained from S. Head at the University of Toronto) were able to bind as well to a glycolipid (GM_1) which did not contain the important Gal(α1-4)Gal disaccharide. These results illustrated that the VT-1 which I obtained was able to recognize receptors in the

iodinated state. Moreover, iodinated VT-2 also appeared to bind to Gb_3. Therefore, my inability to detect binding of VT to the HeLa and Vero cells in the radioreceptor binding assays was not likely a result of iodination damage to the binding domains.

Since I suspected that a means of resistance to the toxin might be due to a generalized inability of the cells to internalize molecules present in the growth media, I wanted to examine receptor mediated endocytosis (RME) in the toxin resistant clones which had normal levels of Gb_3. My reasoning was that the RME process is used in the entry mechanism of toxins such as diphtheria toxin (Middlebrook and Dorland 1981) and may also be involved in the entry of the shiga-like toxins.

Ohkuma and Poole (1978) developed a method that could be used to examine the ability of cells to acidify their endosomes. However, I modified the Ohkuma and Poole procedure by using FITC-EGF in order to make it more specific and increase the sensitivity. I reasoned that the sensitivity of the assay would be increased because much of the FITC-labelled dextran may not be taken into the cell but might remain bound to the outer surface. Therefore, only a small amount of the probe would be internalized and the change in the fluorescence intensity would be lower than if an RME-receptor specific FITC-probe such as FITC-EGF was used. This would increase the ratio of internalized to surface-exposed FITC.

I found that Vero clone cm:17 had a 70% decrease ($p<0.05$) in the rate of fluorescence quenching over the parental strain. This indicated that although not highly significant, in view of the Gb_3 data, the defect in Vero cm:17 may be at the level of RME. This could be due to a problem with the preliminary internalization of the FITC-EGF into the endosome, or to an inability of the cell to acidify its endosome as rapidly as the parental strain. A decrease in the rate of fluorescence was also observed in Vero clone cm:3 ($p<0.1$) but the decrease was not as great as Vero clone cm:17. However, in HeLa clone cm:3 the rate of fluorescence intensity increased ($p<0.1$) in comparison to the parental strain. Apparently, HeLa clone cm:3 does not have a deficiency in its ability to internalize EGF and/or acidify endosomes.

A third mechanism of resistance to a toxin might involve an alteration of the intracellular target for the enzymatic activity. This is observed in cells which have become resistant to diphtheria and *Pseudomonas* exotoxin A. In normal cells, the target for diphtheria toxin is EF2 which is inactivated by ADP-ribosylation of a post-translationally modified histidine residue termed diphthamide. Mutant cell lines which lack the modified histidine are completely resistant to both diphtheria and *Pseudomonas* toxin A (Möehring, *et al.*, 1980). However, in the case of the VTs, I feel that an alteration in the target would probably be a lethal mutation in the cell, as it has been shown by

Ogasawara *et al.* (1988) and Hovde *et al.* (1988) that the intracellular target for VT-1 and VT-2 is a highly conserved region of the eukaryotic ribosome. It is unlikely that a mutation in this region would be compatible with life.

In summary, I have investigated VT-2 resistant HeLa and Vero cells to gain a better understanding of VT-2 receptors. I found that the most resistant toxin clones (HeLa cm:1 and HeLa cm:2) had a 50% reduction in the neutral glycolipid Gb3 which has been proposed as a cell receptor for VT-1 and VT-2. I have also found that Vero cm:3 ($p<0.1$) and Vero cm:17 ($p<0.05$) have a reduction in the rate of endosome acidification, which suggests that the mechanism whereby VT-2 gains access to its intracellular target is through RME. In my investigations, I have not been able to discover a defect in the clone HeLa cm:3 to explain its resistance to VT-2. It is possible that HeLa cm:3 has a defect which I have not been able to evaluate. This clone may be able to internalize the toxin normally via the process of RME, but there may be a defect in an intraendosomal process which is necessary for transporting the toxin to the cell cytoplasm.

5. References

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APPENDIX 1

Phosphate Buffer Solution

To make 10X PBS:

KCl	2 g
KH_2PO_4	2 g
NaCl	80 g
$Na_2HPO_4 \cdot 7H_2O$	21.6 g
or	
Na_2HPO_4	11.44 g

1. Dissolve the above chemicals in double distilled water and make up to the 1 L mark. Autoclave if desired
2. To make 1 L of PBS with Mg^{+2} and Ca^{+2} add 1 mL of 0.1 M of $CaCl_2$ and 10 mL of 0.1 M $MgSO_4$ (these solutions may be autoclaved).

APPENDIX 2

Lowry Assay

(Lowry, *et al.*, (1951) J. Biol. Chem. **193**: 265-275)

I. Solutions

Solution A:

-4 g sodium hydroxide (NaOH)
-20 g sodium carbonate (Na_2CO_3)
-0.2 g potassium sodium tartrate (KNa Tartrate)

Dissolve the above chemicals in distilled water and make up to the 1 L mark.

Solution B:

-0.2 g Cupric sulfate ($CuSO_4 \cdot 5H_2O$)

Solution C: add one part of Solution B to 50 parts of solution A.

II. Assay Procedure:

1. Use 0.1% BSA dissolved in PBS as the working standard solution.
2. To prepare the working standard solutions add 10-60 µg of the standard protein to the respective test tubes and add distilled water to give a final volume of 200 µL.
3. Add to each tube (both tests and standards) 400 µL of Solution C. Incubate the tubes at room temperature for 10 minutes.
4. Add 50 mL of the Folin Reagent (diluted 1:1 in PBS or distilled water).
5. Incubate the tubes a further 30 minutes at room temperature and read the absorbance of each tube at 600 nm.

APPENDIX 3

Laemmli gels

(Laemmli, U. K. (1970) Nature **227**: 680-685)

(Discontinuous gel system)

Separating Gel	12.5%	16.0%
Acrylamide:Bis (30:0.8)	12.5 mL	16.0 mL
1.875 M Tris (pH 8.8)		6.0
0.2 M EDTA	0.3	
Milli-Q ™ Water	10.9	7.4
TEMED		
(add before persulfate)	0.015	
10% Ammonium persulfate	0.3	
	30.015 mL	

5% Spacer Gel

Acrylamide:Bis (30:0.8)	2.5 mL
1.0 M Tris (pH 6.8)	1.88
0.2 M EDTA	0.15
Milli-Q™ Water	10.3
TEMED	
(add before persulfate)	0.008
10% Ammonium persulfate	0.15
	15.0 mL

Laemmli Running Buffer (5 liters Milli-Q™ Water)

Tris (Sigma 7-9)	15.125 g
Glycine	72.0
SDS (BDH or Bio-rad)	5.0

Laemmli Digestion Mixture

10% SDS (BDH)	2.0 mL
Dithiothreitol (DTT)	0.078 g (50 mM final)
-**or**-(β-mercaptoethanol)	(0.4 mL)
glycerol (glycerin)	1.0 mL
1.0 M Tris pH 6.8	0.625 mL
Milli-Q™ Water	6.0 mL
Saturated Bromphenol blue	
(in water)	0.075 mL
	10.025 mL

APPENDIX 4

Continuous Linear Gel System

Seperating Gel	7.5%	15.0%
Monomer (8)	4.24 mL	---
Monomer(9) + glycerol	---	8.48 mL
1.875 M Tris (pH 8.8)	4.25 mL	4.25 mL
0.2 M EDTA	160µL	160 µL
Milli-Q™ Water	8.43 mL	4.19 mL
10% Ammonium persulfate	37 µL	37µL
TEMED	6 µL	6µL

Stacking gel made up as per discontinuous gel

Monomer (8) Solution

Acrylamide	58.4 g
Bis	1.6 g
Milli-Q™ Water	make up to 200 mL

--Store at 4° C in the dark

Monomer (9) Solution

Acrylamide	58.4 g
Bis	1.6 g
75% glycerol	make up to 200 mL

--Store at 4° C in the dark.

APPENDIX 5

EDTA Wash Solution

0.201 g/L disodium EDTA
8.00 g/L NaCl
0.40 g/L KCl
2.86 g/L Trizma Hydrochloride
0.84 g/L Trizma Base

Adjust pH to 7.0. Dispense into glass bottles. Autoclave 121°C. for 20 minutes.

APPENDIX 6

Lifting Buffer and Lysis Buffer for Tissue Culture Membrane Preparations

Lifting Buffer

137 mM NaCl	8.01 g/L
5 mM KCl	0.374 g/L
5.6 mM Glucose	1.01 g/L
1 mM EGTA	0.38 g/L
5 mM HEPES	1.11 g/L

pH 7.4

Lysis Buffer

20 mM Tris (pH 7.5)
5 mM $MgCl_2$
1 mM EGTA
1mM dithiothreitol

APPENDIX 7

Buffer Solutions for the Direct ELISA

Coating Buffer

1.59 g Na_2CO_3
2.93 g $NaHCO_3$
0.20 g NaN_3
1000 mL H_2O

pH 9.6

Phosphate-buffered Saline Tween Buffer (PBST)

8.00 g NaCl
0.20 g $KH_2PO_3 \cdot 12H_2O$ (0.46 g without H_2O; 0.75 g with $7H_2O$)
0.20 g KCl
0.5 mL Tween 20
1000 mL H_2O

pH 7.4

PBST-BSA

To PBST add 1% BSA w/v

10% Diethanolamine Buffer

97 mL Diethanolamine
800mL H_2O
0.20 g NaN_3

Adjust pH to 9.8 with concentrated HCl then make up to 1.0 L

APPENDIX 8

LPS Silver Stain

(Tsai, C. M. and C. E. Frasch. (1982) Anal. Biochem.**119**:115-119)

Reagents:
Oxidizing Reagent: 1.4 g of periodic acid dissolved in 200 mL of 40% ethanol, 5% acetic acid
Silver Stain Solution: Add 2.8 mL of 1.0 N ref NaOH to 25.2 mL dH_2O. Next add 2 mL of concentrated (i.e. 29%) ammonium hydroxide solution with vigorous mixing, slowly add 5 mL of a 0.2 µ filtered solution of 20% wt/vol silver nitrate. A transient brown precipitate should be observed. Add 115 mL dH_2O
Developer: To one L of water add 0.5 mL of 37% formalin solution. Further, add 50 mg of citric acid. (May be stored at room temperature indefinately)

Method:
--Fix gel overnight by gently gyrotory shaking in about 800 mL of 40% ethanol, 5% acetic acid in a chromic acid-cleaned Pyrex baking dish covered with Saran™ wrap. This fixing/washing step is essential to remove SDS which will give a murky yellow background to the gels if it is not removed.

--Pour off fixer and add 200 mL of oxidizing reagent. Allow to react, with mixing, for 5 minutes.

--(OPTIONAL) transfer gel (use well rinsed gloves) to a second acid-cleaned dish containing approximately 800 mL deionized water.

--Wash gel three times for 15 minutes each with 800 mL deionized water.

--After last wash, pour off water, add entire 148 mL of silver stain reagent and allow to react with gel, with gyrotory mixing, for 10 minutes.

--Pour off silver stain reagent and wash as before with deionized water. Wash three times, changing the water every 10 minutes.

--Add about 400 mL of developer and allow bands to appear to desired intensity, or until background begins to yellow. Stop development by adding 7% acetic acid. Store in 7% acetic acid away from light, since bands will fade upon prolonged exposure to room light.

--**NOTE:** This silver stain is suitable for proteins, too. Just eliminate the step involving periodic acid oxidation.

APPENDIX 9

Preparation of McFarland Nephlometer Standards
(National Committee for Clinical Laboratory Standards. 1983. Methods for dilution anitmicrobial susceptibility tests for bacteria that grow aerobically. M7-T 3:31).

-Set up 10 test tubes or ampules of equal size and of good quality. Use new tubes that have been throughly cleaned and rinsed.
-Prepare 1% chemically pure sulfuric acid.
-Prepare 1% aqueous solution of chemically pure barium chloride.
-Add the designated amounts of the two solutions to the tubes as shown below to make a total of 10 mL per tube.
-Seal the tubes or ampules. The suspended barium sulfate precipitate corresponds approximately to homogenous Escherichia coli cell densities per milliliter throughout the range of standards, as shown below.

	TUBE NUMBER									
	1	2	3	4	5	6	7	8	9	10
Barium chloride (mL)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.1	1
Sulfuric acid (mL)	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approximate cell density ($\times 10^8$ /mL)	3	6	9	12	15	18	21	24	27	30

APPENDIX 10

Brom Thymol Blue ("B") Broth for *Ureaplasma*

Robertson, *et al.* (1975) J. Bact. **124**(2): 1007-18

Basal Medium

Glass distilled water	90.0 mL
PPLO Broth w/o CV (Difco Cat. No 055401	2.1 g
Yeast Extract (Difco Cat. No. 0127-01)	0.1 g
Brom thymol blue solution (0.4%)	1.0 mL

Autoclave; when cool, add:

Required supplements	volume	final concentration
Sterile horse serum (pH 6.0)	10.0 mL	10%
Urea solution (10% v/w)	0.25 mL	0.025%
Glycyl-L-histidyl-L-lysine acetate solution (Calbiochem Cat. No.363941-20 µg/mL)	0.1 mL	0.02 µg/mL

Optional Supplements (for primary isolation):

Ampicillin solution (100 mg/mL)	1.0 mL	1 mg/mL
Nystatin solution (50,000 U/mL)	0.1 mL	50 U/mL
Lincomycin solution (20 mg/mL)	0.1 mL	20 µg/mL

Ater all supplements have been added, adjust pH to 6.0 with 1N HCl. Solutions for supplements and complete medium should be kept at -20°C.

Colour Key

pH 6.0 yellow
pH 6.8 chartreuse (about 10^7 ccu/mL)
pH 7.2 green (about 10^8 ccu/mL)

Media for Mycoplasma

"Standard" Broth

PPLO broth base	2.1 g
Yeast extract	0.1 g
HEPES buffer	1.19 g (optional)
Distilled water to	80.0 mL

Autoclave

When sufficiently cool add:

Horse serum	20.0 mL
Ampicillin (100mg/mL)	1.0 mL

Adjust pH to between 7.6-7.8

"Standard" Agar

PPLO agar base	3.5 g
Yeast extract	0.1 g
Distilled water to	80.0 mL

Dissolve agar by heating solution.
Autoclave.
When sufficiently cool add:

Horse serum	20.0 mL
Ampicillin (100mg/mL)	1.0 mL

Adjust pH to between 7.6 and 7.8

APPENDIX 11

Procedure Used to Prepare Immunoaffinity Column

Axen, *et al.* (1967) Nature. **214**: 1302-1303.

1. Weigh out the required amount of CNBr-activated Sepharose 4B (1 g freeze-dried material=3.5 mL final gel volume).

2. Wash and reswell the gel using (200 mL/g of gel) 1 mM HCl on a sintered glass filter.

3. Dissolve the protein to be coupled in 0.1 M $NaHCO_3$ buffer pH 8.3. which contains 0.5 M NaCl ("coupling buffer") (5-10 mg protein :1 mL gel).

4. Mix the protein solution with gel suspension end over end for 2 hours at room temperature or overnight at 4°C. Do not use a magnetic stirrer.

5. Wash away the excess protein with coupling buffer. Block the remaining active groups with 1 M ethanolamine solution pH 9.0 for 2 hours at room temperature.

6. Wash away excess blocking reagent and adsorbed protein with coupling buffer, followed by 0.1 M acetate buffer pH 4.0, followed by coupling buffer.

7. The protein-Sepharose is now ready for use. Store at 4°C.